

End of the road for poliomyelitis?

There seems a real prospect that one of the infectious scourges of the past half century will not persist much beyond the present century, but polio will be only the second infection of human beings to be eradicated. Many more remain.

WORLDWIDE, the number of new cases of poliomyelitis this year is unlikely to exceed some four-digit number, two orders of magnitude less than the incidence of the disease just two or three decades ago. That is what the World Health Organization (WHO) is now saying. WHO also says that the infection may well be eliminated early in the next century, perhaps even sooner. On the heels of the eradication of smallpox in 1994, that will be something for everybody to boast about. But before we throw our hats in the air in celebration, we should not forget that these developments are also a salutary reminder of the obduracy of infectious diseases, with which we and our children will have to battle for decades to come.

The history is instructive. The parasitism of poliovirus on human beings may well go back as far as *Homo sapiens*, but would have mostly been concealed by the enhanced susceptibility of those infected to more common (or more recognizable) infections. Childhood leukaemia, apparently newly emergent in the 1950s, was similarly an artefact of the widespread use of antibiotics for the treatment of the infections from which leukaemic people previously died. The battle against polio was mounted in the United States by a private charity called the March of Dimes, itself a response to the general sense of shock that even hygienically trained people might be maimed or even killed by a virus commonly transferred in ordinary water. By the 1930s, the 'iron lung' had become a symbol of human frailty; some emerged intact or only maimed, others not at all. Even the President of the United States, Franklin D. Roosevelt, was a polio victim. The March of Dimes set its sights on prophylaxis, and eventually succeeded, although not without setbacks.

Jonas Salk's first vaccine, based on the use of killed preparations of virus, killed many of its late-1950s recipients because not all the virus particles had ceased to be viable. Luckily, the second string to the charity's bow, the attenuated but live virus developed by Sabin, turned out to be as effective, safer and even more convenient. (Injections were not necessary.) That 'oral' vaccine, administered on pieces of sugar or even blotting paper, has been the chief instrument of the near-eradication of polio. Even so, it will have been nearly half a century between the introduction of the Sabin vaccine and the practical eradication of the causative virus. (Jenner's smallpox vaccine had to wait two centuries for the same proof of success.)

There are several lessons to be learned. First, it is always much easier to recognize the virus responsible for a known infectious disease than to develop a safe and effective vaccine. (The contemporary case of AIDS refers.) Second, the cost of developing vaccines is inevitably high, but much less than the capital value of the death and disability the infections cause. But smallpox and now poliomyelitis show that vaccine manufacturers will eventually put themselves out of business. Coupled with manufacturers' legal responsibility for the vaccine shots that damage their recipients and the circumstance that the most urgent needs are frequently those of impoverished populations in the developing world, it is no wonder that vaccines are the pharmaceutical industry's orphans. Yet the need for protection against bacterial and viral infections as different as tuberculosis and hepatitis-B (and E) is clamant and persistent. Malaria and the other parasitic diseases are something else again.

It is in everybody's interest that poliomyelitis should not be the last infection to be eradicated. Indeed, the techniques of genetic engineering now offer the hope that new vaccines may be built around synthetic peptides that mimic the antigenic properties of intact microorganisms (but it is puzzling that peptides are often so much less effective than expected). But that is only one of the several routes to more effective prophylaxis suggested by the better understanding of human immunity now being brought to light. In other words, there is good reason to hope that development costs will eventually be reduced, and that infectious microorganisms not now lending themselves to immunization will be brought within the net.

But who will take charge of that endeavour? The World Health Organization has fiscal mechanisms for tackling these problems, but insufficient funds to do much and too cumbersome a bureaucracy to act quickly. It is interesting that the new commissioner for research at the European Commission, Mme Edith Cresson, is advocating that vaccine development should be one of several initiatives supported by the commission's current Framework programme. Although commission-watchers will be disconcerted by this further evidence that each new research commissioner regards his or her appointment as an invitation to change the direction of Europe's centrally supported research, there is little doubt that this particular innovation could be of great benefit for the world at large. □



South African scientists urged to propose projects for future support

Johannesburg. A leading official of South Africa's new democratic government has appealed to the nation's scientists for help in developing its science and technology investment programme.

Bernie Faranoff, the official charged with South Africa's Reconstruction and Development Programme (RDP), said the government is ready to invest in science, but needs advice in picking the right areas.

Speaking at a conference held at the University of the Witwatersrand in Johannesburg and organized by the Royal Society of South Africa, Faranoff said that the RDP recognized the need to develop the human

resources needed by science and technology.

But he added that "it is up to the universities and scientists themselves to come up with suggestions on how they can contribute to development in South Africa". Government spending on research and development (R&D) in next year's budget, he said, would be determined by the RDP office in line with the plan's goals, along with budgets for all government departments.

Faranoff, a radio-astronomer by training who spent nearly two decades working in the trade union movement, has in the past said he believed spending on R&D in South Africa should be "drastically increased". He told the meeting, which was opened by Ben Ngubane, Minister of Arts, Culture, Science and Technology, that the national development plan being drawn up would support good proposals for developing science and technology in South Africa. But, he added, it was important that the proposals arose from a partnership of scientists in universities, government departments and industry.

Acknowledging the relatively small sums allocated for science in this year's budget — allocations to individual research councils have been largely calculated by awarding small, if varying, percentage increases to each (see *Nature* 374, 487; 1995) — Faranoff promised that next year's budget would be "zero-based", with funds allocated in accordance with the goals of the RDP.

This would be the first phase of a multi-year funding programme, he said, whose length is yet to be decided by Cabinet. Such an approach, he explained, would lend stability to the system by ensuring funding for the whole duration of research programmes — provided that agreed performance criteria were met.

The government is expected to complete by August an audit of its present allocation and use of all state funds, including those handed out for research. The object of the exercise is to assess the extent to which funds contribute to the RDP.

South Africa's cumulative science budget has had to withstand major changes since 1989, with defence R&D spending being the key casualty.

Out of a total current R&D expenditure of R1,645 million (US\$454 million) — just over half of which is distributed among the seven research councils — R220 million goes to the Department of Defence for military research, with a further R490 million paid as a subsidy to the Atomic Energy Corporation (AEC), for enriching uranium used in South Africa's nuclear weapons.

The remaining money is distributed to

the Department of Environmental Affairs' Sea Fisheries Research Institute, the Water Research Commission, the Department of Health's research institutes, various conservation agencies, and to groups carrying out non-nuclear energy research.

In 1989, by contrast, defence R&D spending — R1,500 million — took the lion's share of the total R&D budget. A further R1,000 million went to the AEC. Doubling these sums to make them comparable to 1995 prices indicates the extent of the decline in state spending on R&D over the past six years as a result of the decline in its military component.

The Witwatersrand meeting's main function was to debate a draft policy suggestion prepared by George Ellis, president of the Royal Society of South Africa (see *Nature* 371, 94; 1994). Participants split up into groups to discuss specific proposals for physics and chemistry, earth sciences, life sciences, mathematics, and environmental sciences as well as interdisciplinary research.

Within the life sciences, participants agreed on three areas of importance. The first included research on aspects of health, agriculture and the environment which address basic human needs, one of the main themes of the development programme.

Identifying areas of life sciences research which could stimulate growth in the South African economy — the other principal theme of the RDP — proved more challenging. Two suggestions, both related to the country's uniquely-rich biota, were to investigate ways of exploiting indigenous knowledge of naturally-derived medicinal products, and to promote research on biodiversity in order to boost the country's burgeoning efforts in 'eco-tourism'.

Finally, the participants in the life sciences group felt that existing scientific strengths should be built on, regardless of their potential utility.

Michael Cherry

Nature ownership to change hands

London. Macmillan Ltd, the privately owned British publishing group whose subsidiary, Macmillan Magazines Ltd, is the publisher of *Nature*, announced last week that the German publishing house, Verlagsgruppe Georg von Holtzbrinck, is to take a majority stake in the company.

Holtzbrinck, which was founded in 1971, has a range of publishing and media interests, including the US publishers Henry Holt, and Farrar Straus and Giroux. Its imprints include the academic publishing houses Spektrum Akademischer in Germany and W.H. Freeman and Worth in the United States, and it is also the publisher of *Scientific American*.

Under an agreement between the two companies, Macmillan — which launched *Nature* in 1869 — will continue to operate independently, and a statement from the company says that no changes in the management or the organization of the business are planned other than those that will evolve through its continuing expansion.

The board of Macmillan, most of whose shares are held in trust for the Macmillan family, had previously considered a public flotation. But Nicholas Byam Shaw, its chairman, said that this had been rejected "in favour of an alliance with a publisher whose philosophy of investment matches the long-term nature of Macmillan's own culture and tradition".

Sir John Maddox, the editor of *Nature*, said that he welcomed the move as "probably the best for *Nature*" of the various options that had been under consideration by the board. "Holtzbrinck is a company with a long-term view of the health and welfare of magazines, which it has already shown in its treatment of other titles," he said. □

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Europe sets hopes on twin eyes in the sky

Paris. Scientists across the world will be keeping their fingers crossed tomorrow night (21 April) when ERS-2, the European Space Agency (ESA)'s latest remote-sensing satellite, takes its place in orbit alongside its ageing predecessor, ERS-1.

Any accident or delay in the launch of the second satellite would spoil a unique opportunity to carry out new research that is only possible using both satellites in tandem.

ERS-2 was originally designed merely to take over the baton from ERS-1, which was launched in 1991. But at the end of 1993, when it became clear that ERS-1 was likely to be able to continue operating beyond its 30-month design life, users asked the space agency to consider operating both satellites in tandem (see *Nature* **370**, 317; 1994). The extra costs were formally approved by the ESA council last month.

Like ERS-1, ERS-2 is equipped with a radar altimeter, which gives precise measurements of the heights of the oceans and ice sheets, and a synthetic aperture radar (SAR), which provides images with a resolution of 10 centimetres.

The tandem mission will allow full exploitation of the SAR's unique ability to carry out interferometry — the comparison of two pictures of the same area taken at different times — and will also provide a precise digital three-dimensional map of the Earth's land surfaces.

But to place ERS-2 in the appropriate orbit, Arianespace has just a four-minute window to launch the satellite, to be carried on an Ariane IV launcher from ESA's space centre in Kourou, French Guiana.

Moreover, following launch failures in January and December last year, Ariane-space now has a backlog of 38 launches. If the launch of ERS-2 is postponed, it may be

ESA

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ERS-2's GOME will measure atmospheric ozone in blocks of 40 by 80 kilometres.

difficult for the company to find it another slot in its planned hectic schedule of one launch every three weeks.

Apart from assuring the continuity of the ERS programme and working in tandem with its predecessor ERS-1, ERS-2 will have an additional three channels in its scanning radiometer, allowing it to track changes in vegetation cover in great detail.

The satellite will also carry a new instrument to measure the atmosphere's ozone content. GOME (Global Ozone Monitoring Instrument) as it is known, marks a major advance on existing US and Russian satellite-borne ozone-measuring instruments.

GOME, in particular, possesses a broader spectral range (240–790 nm), than existing instruments, which work only in the ultraviolet (UV). This will allow it to take measurements in the polar regions in late winter, when the Sun is too low for ultraviolet light.

Compensating for this blind spot in existing instruments at present, requires the release every year of stratospheric balloons over Northern Europe. These balloons give only a part of the overall ozone picture. GOME should provide ozone maps of the entire globe every three days.

ESA officials are being cautious, however, about overselling GOME's potential, given the instrument was hurriedly built in only five years — complete validation of the instrument would have taken ten years of development and testing. "We cannot give a 100 per cent guarantee that GOME will live up to its expectations", one official says.

The remote-sensing satellite programme is widely regarded as one of ESA's most successful science missions. The number of users of ERS-1 data is growing at 25 to 30 per cent annually, and the space agency has received more than 15,000 requests for data. More than half the data go free of charge to 270 selected research teams; seven per cent of the data is bought by commercial users at list price.

By reusing technologies developed for ERS-1, ESA has been able to build ERS-2 for ECU550 million (US\$714 million), 60 per cent of the cost of ERS-1. The new satellite will operate from 1995 to 1998, when it will be replaced by Envisat. **Declan Butler**

Patent convention 'needs new protocol on moral issues'

London. A new protocol should be drafted to the European Patent Convention, setting out in detail how its clause specifying when patents cannot be granted on moral terms should be interpreted, according to a top-level British bioethics advisory committee.

In a report to be published this week on the ethical and legal issues raised by the use of human tissue in medical treatment and research, the Nuffield Council on Bioethics — widely seen as the United Kingdom's national bioethics council — urges the government to press its co-signatories of the convention to negotiate such a protocol to clear up current confusion over the clause.

The report also suggests changes in legislation in order that, under strict safeguards, tissue can be removed for non-therapeutic research purposes from 'incompetent adults' — for example, those suffering from Alzheimer's disease — who may be incapable of fulfilling current requirements for providing informed consent.

The report has been prepared by a work-

ing party consisting of medical, ethical and legal experts. It was chaired by Dame Rosalinde Hurley, professor of microbiology at the Royal Postgraduate Medical School.

Citing the recent dispute between the University of California and a patient, John Moore, over the ownership of cell lines taken during routine medical procedures and subsequently used in a commercial procedure, Sir Patrick Nairne, the chairman of the council, says that the uses of human tissue in both therapy and research are presenting "more, and more difficult, problems of ethics and law."

On the question of patents, the report says that a body of opinion would like clarification of the EPC clause that excludes patents that contravene "public order or morality". Given the European Parliament has recently rejected attempts by the European Commission to draft a directive aimed at achieving this goal (see *Nature* **374**, 103; 1995), the report says that "the preferred course may be an amendment to or clarification

of the EPC itself."

"It might be helpful if governments could agree on some concepts which might guide national courts in deciding these issues," says Hurley. She adds that the working party was less enthusiastic about other possible courses of action — such as the creation of a Europe-wide ethics committee concerned exclusively with the resolution of ethical issues in patent applications.

As far as issues involving informed consent are concerned, the working party says that — in order to avoid the type of dilemma raised by the John Moore case in the United States — the law should proceed on any claim over removed tissue by looking closely at the basis of the consent that was given to the removal procedure.

"In particular, it should be regarded as entailed in consent to medical treatment that tissue removed in the course of treatment will be regarded as having been abandoned by the person from whom it was removed," says the working party. □

Election rivals promise French R&D boost

Paris. When French voters go to the polls this weekend in the first round of France's presidential elections, most will be indifferent to research. But the seven-year term of office of the winner of the final round, to be held on 7 May, will have a major impact on the shape in which French science enters the next millennium.

Research has not figured prominently during the campaign. This is hardly surprising, given that few issues have received serious debate in a campaign dominated by fratricidal squabbling between former prime minister Jacques Chirac, and the present incumbent, Edouard Balladur, both candidates for the Gaullist RPR party.

Of the three leading candidates, only Lionel Jospin, the Socialist candidate, has spelled out his detailed policy on research. But all claim that they would make research a national priority. Both Balladur and Chirac have promised to increase spending on science from 2.4 per cent of gross national product (GNP) to match that of the United States and Japan, currently around 2.8 per cent; Jospin has set an even more ambitious goal of 3 per cent.

But many researchers are sceptical that the claims by Balladur and Chirac amount to more than campaign rhetoric. Indeed, many argue that the traditional differences in the research policies of the left and right are alive and well.

While the modernization of French science began with the Gaullist government of 1958, largely because of preoccupations with defence and the nuclear industry, the tone of the last decade has been set by the enthusiastic policies of the socialist government elected in 1981 under President François Mitterrand.

Mitterrand not only declared research a national priority, but ensured that funding subsequently grew from 1.87 per cent of GNP in 1981 to 2.4 per cent in 1990. Hubert Curien, who was science minister for much of the decade, developed a coherent long-

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Jospin (left), the Socialist party candidate, has been more specific than his right-wing rivals Balladur (right) and Chirac (centre).

term policy, increased recruitment, prodded research organizations to become more efficient, and encouraged industry to do more research through tax credits and subsidies.

In contrast, memories remain fresh in the scientific community of the harsh cuts on research imposed by the Chirac-led government which held power between 1986 and 1988, and argued, as part of wide cuts in public spending, that industry should bear more of the burden of funding research.

Similarly, some claim that much of the progress made under the socialists has been undone under the Balladur government, which came to power in 1993. While research has not suffered cuts as brutal as between 1986 and 1988, funding has stagnated and recruitment has slowed — despite the scheduled retirement of half of all French researchers by 2005.

Many of the initiatives launched by François Fillon, the minister for higher education and research, have either failed to materialize or ended in climdbowns. In 1993,

Fillon was forced to abort his attempts to reform the university system, for example.

Similarly, a six-month 'national consultation' on research, expected to result in a government white paper setting out a new national strategy for research, has produced few changes.

Associated Press

Many researchers see Jospin as having the most legitimate credentials as a defender of research. His approach to university research and higher education while minister for national education in the previous socialist government is widely considered as having been energetic and progressive, if sometimes controversial.

What sets Jospin apart from the other candidates, say some

observers, is that he has a genuine enthusiasm for research. This is attributable in part to the company he keeps; his main adviser and close friend is Claude Allègre, head of the Institut du Physique du Globe at the University of Paris VI, and formerly Jospin's director of higher education.

In Jospin's current campaign, issues concerning research and higher education are being handled by another geophysicist, Vincent Courtillon, who heads the Laboratory of Palaeomagnetism and Geodynamics on the same campus as Allègre, and is chairman of the European Union of Geosciences.

Courtillon says that "independent evaluation" would be a key element of Jospin's research policies. In particular, he would set up committees made up of both French and foreign experts to evaluate all government policies, including space.

Jospin would also increase recruitment of young researchers to the Centre Nationale de la Recherche Scientifique (CNRS), France's largest research organization, and introduce a three-year plan to redress the agency's financial crisis (see below). But recruits would be discouraged from pursuing their entire career within CNRS.

In a bid to encourage greater mobility, one-third of such recruits would be encouraged to transfer to the universities after 15 or so years at CNRS, and another third to industry, according to Courtillon.

The election of Balladur would not mean a "revolution" in research, according to Thierry de Mazencourt, his campaign adviser on industrial, commercial and research issues. "The system is not catastrophic", he says. What it needs are many low-profile improvements.

Balladur's main priority, says de Mazencourt, would be to improve links between industrial and fundamental research. In particular, he would create a series of "regional networks for technological diffusion".

Declan Butler

CNRS budget crisis 'larger than admitted'

Paris. Concern is growing that a funding crisis at France's largest research agency, the Centre National de la Recherche Scientifique (CNRS) may be much more serious than the government has admitted, and that government ministers may be unable — or unwilling — to resolve the problem.

The concern has been heightened by doubts over a recent promise by François Fillon, the research minister, to reimburse a FF500 million (\$100 million) deficit which the agency has accumulated over the past few years because payments from the state have failed to keep up with promises made in the annual budget.

In a statement released last week, several CNRS researchers claimed that the terms of the letter from the government to

CNRS formalizing this promise did not explicitly commit it to reimbursement of the debt, but rather provided an advance on this year's payments to the agency. They want an explicit and binding commitment by the government that would remain valid whoever wins the upcoming presidential elections.

Moreover, an official audit of CNRS finances will be made public next month, and sources familiar with the investigation say that the CNRS deficit may not be FF500 million, but as much as FF2.5 billion. The budget for laboratory equipment and supplies is reported to make up FF1 billion of this debt, equivalent to an entire year's allocations. A further deficit of FF1.5 billion is said to have accumulated in the salary budget.

D. B.

Patent office sets firm guidelines on protection of plants

Munich. Plants can be patented, according to a landmark decision published by the European Patent Office (EPO) last week — provided that they are the product of a wholly microbiological process. Furthermore, in order to demonstrate that they contravene “public morality”, and thus cannot be patented, critics must show that biotechnological inventions are harmful to society or to the environment.

Both rulings, which involve crucial interpretations of the European Patent Convention, have been offered as part of a lengthy explanation by the EPO's board of appeals for its decision in February that a patent covering a genetic engineering process for producing herbicide-resistant plants cannot cover the plants and seeds resulting from this process (see *Nature* 374, 8; 1995).

Patent lawyers claim that the appeal court's rulings have, for the first time, provided clear guidelines on what will and what will not be accepted for patenting in plant biotechnology, eliminating much of the confusion caused in particular by the convention's explicit ban on the patenting of new plant varieties.

The rulings relate to a broad patent granted by the EPO in 1990 to the Belgian company Plant Genetics Systems and the US biotechnology company Biogen Inc. for plant cells produced through genetic engineering which are resistant to glutamine synthetase inhibitors. An appeal was subsequently made against the patent by the environmentalist group Greenpeace.

In February, the Technical Board of Appeal ruled that although a patent could be granted for a method of producing herbicide-resistant plants and seeds, it could not be granted on the plants and seeds themselves. In explaining this decision, the board now says that it rejected Greenpeace's claim that the process contravened “public morality” since, despite the environmentalist group's concerns, there was no clear evidence that the plants produced would result in harm to the natural environment.

In describing why it had agreed to allow the patents to be granted on the plant cells arising from the process, the board said that such cells “cannot be considered to fall under the definition of a plant or of a plant variety”, noting further that “plant cells are considered to be ‘microbiological products’ in the broad sense under the current practice of the EPO.” Such products are not excluded from patenting by the convention.

“This decision is a major victory for the biotechnology industry, providing the industry with the legal security for future research and planning,” one patent attorney commented last week. □

White House rejects proposal for Department of Science

Washington. Jack Gibbons, President Bill Clinton's science advisor, has rejected proposals for a new Department of Science, and fiercely criticized recent congressional action on the science and technology budget, which he has branded “a ruthless attack on this nation's future”.

In an uncharacteristically forthright attack on the Republican programme, made at the annual science budget forum organized by the American Association for the Advancement of Science last week, Gibbons said: “My hope is that wise heads in Congress will intervene, and save the nation from permanently damaging our research and development base. But my fear is that history will record that extremists in Congress prevailed in an atmosphere of budget chaos, driven by a fundamental disregard for reinvestment in science and technology.”

Cuts already imposed by Congress in the current 1995 financial year “read like a litany of the lost,” Gibbons said. But Republican congressional staff warned the budget forum to expect far more substantial budget cuts for the 1996 financial year. Harlan Watson, staff director of the energy and environment subcommittee in the House of Representatives, told the AAAS that the discretionary budget — of which science and technology is a substantial part — would face cuts of 20 to 25 per cent.

Roscoe Bartlett (Republican, Maryland), expressed concern that top research universities, such as Johns Hopkins in Baltimore, were so dependent on the government for support. “We need more money for basic research, but 90 per cent of it should not come from the federal government,” he said.

Bartlett also said that government laboratories should not be “usurping” university research money, and called for something akin to the military's base-closure commission to rationalize these laboratories.

Many of the thousand-or-so science policy specialists who attended last week's forum were surprised by its level of partisan rhetoric, and alarmed by the likely consequences for science funding. Several said that the division between Democrats and Republicans on science policy had never before been so deep, or so public.

In rejecting the Department of Science proposal, Gibbons said that US science relied upon pluralism of support to ensure that good ideas were funded. He also argued that the proposal would divorce science from the agencies, such as the Energy Department and the Environmental Protection Agency, whose missions it is supposed to support. “This administration unequivocally opposes the creation of a Department of Science of the kind now being discussed

in Congress,” he said.

The Department of Science proposal has been championed by Robert Walker (Republican, Pennsylvania), who claims that it could eliminate 5,000 administrative jobs and provide a home for the nuclear weapons programme if the Department of Energy is abolished (see *Nature* 374, 201; 1995).

Describing proposed Republican cuts as “across the board salvos” that could “wreak havoc throughout the research enterprise,” Gibbons cited a recent list of “illustrative” cuts produced by John Kasich (Republican, Ohio), chairman of the House budget committee, which included \$2.5 billion from the National Institutes of Health over five years.

He added that Neal Lane, the director of the National Science Foundation (NSF) “had been told to expect at least a 20 per cent cut”. NSF officials say this is untrue: last month, a House subcommittee chairman asked the agency, and all others under his jurisdiction, to say what the consequences of such a cut would be (see *Nature* 374, 294; 1995).

The Republican majority in the House of Representatives is likely to present its own budget proposal in the second week of May, and the House and Senate will complete their 1996 budget bills in September. In theory, the president can veto budget bills, but House leaders have threatened to bring the government to a halt if he tries to do so.

Colin Macilwain

Earth Day is make or break for greens

Washington. The Clinton administration and environmental groups are using this weekend's celebrations of the 25th Earth Day — 22 April — to try to rally the public in defence of environmental rules and regulations, now under attack in the Republican-controlled Congress.

On Tuesday, vice-president Al Gore launched the administration's National Environmental Technology Strategy. The document charts environmental progress made in the US since the first Earth Day in 1970 and sets some goals for the fiftieth one, in 2020. It sets no new priorities for environmental research, but promises to review them in six month's time.

Some thirty US environmental groups are also planning to use Earth Day to launch one of their largest-ever public relations campaigns against Congress's plans to soften environmental regulation. In particular, the groups oppose risk assessment legislation which has been passed in the House and is now under consideration in the Senate.

C. M.

Earth-bound rules threaten quest for extraterrestrial life

Boston. Astronomers at the Ohio State University who have been engaged in the world's longest running search for extraterrestrial intelligence (SETI) are trying to save their radiotelescope — one of only a handful of such facilities dedicated to SETI activities — from eviction.

The threat to the university's so-called 'Big Ear' telescope comes on top the loss of its grant support from the National Aeronautics and Space Administration (NASA), whose budget for SETI was eliminated by Congress last year.

Since then, existing SETI projects have scrambled to find alternative funding sources. For the Ohio State radio survey, which began in 1972, this has been a double blow, as the 10-year lease for the Big Ear telescope — one of the largest radiotelescopes in the world — expires on 30 July. The owner of the property wants the tele-

scope to be torn down to make room for the expansion of a golf course and the construction of new homes.

paint chips in their homes, may end up killing our telescope. It's a unique dilemma in the history of astronomy."

The Planetary Society has asked its members, and others concerned about the fate of Big Ear, to express their support by writing to the president of the university, E. Gordon Gee. Many have done so, including Carl Sagan, Arthur C. Clarke, and Paul Horowitz, a Harvard physicist.

"The Ohio State University team has been a pioneer in SETI, responsible for many innovations in radiotelescope design," says Horowitz, who heads his own Megachannel Extraterrestrial Assay (META) SETI project at Harvard. "It would be a pity for such a venerable and high-quality group to have to close its doors."

Unlike its Ohio State counterpart, the future of META is relatively secure, thanks to donations from the Planetary Society, the Bosack/Kruger Foundation and other sources. "Private funding is much more stable [than federal funding] when you can get it, because it is not subject to the whims and vagaries of Congress," Horowitz says.

His own programme has recently benefited from another windfall. Micron Technology Inc., the largest manufacturer of computer memory chips in the United States, has agreed to donate 3,420 megabytes of memory chips — worth over \$100,000 — if Planetary Society members can match this contribution.

Once the memory chips are installed later this year, META will become BETA — for Billion Channel Extraterrestrial Assay — as its receiver will be able to analyse a quarter of a billion radio channels simultaneously.

The upgrade will lead to a thousandfold improvement in search capability, and BETA will then be able to scan more channels over a wider range of frequencies than any other SETI effort. Nevertheless, Horowitz believes that with the enormity of the challenge — sifting for evidence of an advanced civilization among 400 billion stars in the Milky Way — it is important to keep several programmes alive, especially those using different search strategies.

"SETI is seriously underfunded worldwide, given that the detection of just one signal of extraterrestrial, and artificial, origin would be the greatest discovery in the history of the human race," he says.

Robert Dixon agrees. "This is one of the most important questions that humans have sought to answer, and we've barely scratched the surface." Dixon acknowledges, however, that the task will require patience and perseverance. "We've known from the beginning that this would be a difficult problem to solve."

Steve Nadis

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Mystical madonna: will science reveal the origins of mystery blood?

DNA evidence called to check 'holy tears'

London. The city of Rome is expecting later this month to host another meeting between the diverse worlds of science and religious belief, this time on slightly different terms than celebrated encounters of the past.

Rather than being castigated as heretics, the researchers in question — forensic scientists from the US Federal Bureau of Investigation (FBI) — have, at least according to the Italian authorities, been invited to use DNA analysis and other contemporary techniques to authenticate the bizarre case of a figurine of the Virgin Mary, said to be weeping "tears of blood".

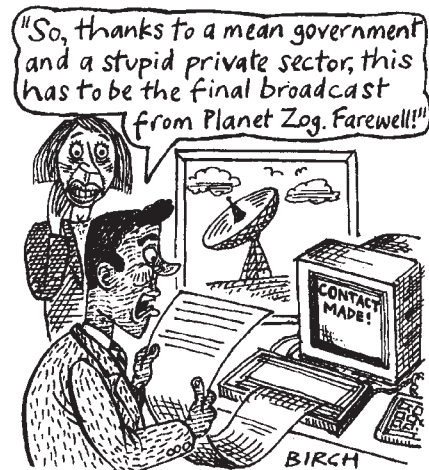
Last week, Cardinal Girolamo Grillo, Bishop of the port of Civitavecchia, northwest of Rome appeared on Italian television claiming to have witnessed a mass-produced statue of the Madonna shedding tears of red liquid after morning mass. "It must be a miracle. They are surely the tears of Jesus," the Cardinal proclaimed.

But one particular viewer was not convinced. Suspecting the work of a charlatan, a city magistrate, Antonio Albano, impounded and locked up the figurine, which had been bought by an electrical worker during a visit to the famous shrine of Medjugorje in the former Yugoslavia.

Doctors at Rome's Gemelli Polyclinic hospital — where the Pope receives treatment — have confirmed that the liquid is male blood. Albano says he has now asked the FBI to carry out DNA tests comparing the tears with the blood of the statue's owner, Fabio Gregori, and his entire family.

The Vatican has so far maintained a diplomatic silence on the affair. But Grillo is said to be furious at the implication that science is being used to measure the integrity of a man of God. "The blood is masculine, so it has to be the tears of Jesus," Grillo told an Italian newspaper. "They have impounded the Holy Madonna and her Bishop is being treated like a criminal suspect." The FBI, meanwhile, says that no invitation to carry out a 'scientific' analysis of the tears has been officially received.

E.M.



scope to be torn down to make room for the expansion of a golf course and the construction of new homes.

"The timing is bad, with our lease coming up for renewal shortly after the cut-off in federal funds," explains Robert Dixon, who directs the Ohio State SETI programme. "When the university asks us what [outside] grants we have, we're forced to say none."

In principle — and assuming that the university continues to pay for rent, electricity and bare upkeep — the all-volunteer project would still be able to keep-going without any outside funding, as it has often done in the past. But there remains a further obstacle.

A provision in the lease stipulates that the telescope must be painted. Federal lead paint laws will make this a difficult and costly job running into \$100,000 or more — an expense the university may not be willing to defray. "It's a ridiculous situation," Dixon observes. "Environmental laws, which were designed to keep small children from eating

Warnings raised over black-market vaccines

London. Unicef, the United Nations Children's Fund, is investigating the large-scale theft and black market resale in Burma of expired and potentially toxic vaccines originally intended for Burma's child immunization programmes.

John Gilmartin, head of purchasing for the agency's immunization programme, who is based in Copenhagen, describes as being "of serious concern" reports sent to him by Unicef's Swiss vaccine suppliers of the existence of more than 10,000 doses of unrefrigerated vaccines on sale in an open market in the Burmese capital, Rangoon.

The vaccines are understood to have disappeared from a Unicef warehouse in Burma and to have reappeared later in Rangoon's Mingala Market. The market is a source of cheap drugs and pharmaceutical products, regularly used by local doctors and patients unable to buy drugs at cost price, but often past their expiry date.

Gilmartin has demanded an explanation from Unicef's local representative, Tiruneh Sinnshaw, who has written to the Burmese Department of Health asking them to intervene, to take the vaccines off the market and to "remedy this dangerous situation".

But Stanley Cryz, technical director of the vaccine suppliers, Swiss Serum and Vaccine Institute, who alerted Unicef to the situation, claims that the agency is not acting fast enough. "This is a very serious situation," he says. "Children's lives are at risk, but Unicef seem to be blasé about the whole thing; our representative went to check just a few weeks ago, and the vaccines are still on sale."

The news of the black-market vaccines has shocked researchers. Barry Bloom, of the Howard Hughes Memorial Institute Research Laboratories in New York, said the whole situation is a catastrophe. "Somebody should go to jail for this," says Bloom. "There is a clear lapse in the system somewhere; it needs to be plugged."

Local physicians estimate that between 50 and 60 per cent of Unicef's vaccines end up on the black market. "As a major supplier of vaccines to Unicef, we became concerned with reports surfacing from several South-East Asian countries that vaccine lots manufactured at our institute solely for the agency were being resold to the public through black markets," says Cryz.

A representative of the institute noticed



Caveat emptor : vaccines on sale in Burma.

during a visit to Burma a painted sign-board inside the Mingala Market advertising the sale of 'Purified duck embryo rabies vaccine', manufactured by the 'Swiss Serum and Vaccine Institute, Berne' (see illustration). He bought two entire lots and despatched them to Switzerland for testing.

According to Cryz, shipping records confirmed that both lots of vaccines — one for tetanus and the other for diphtheria — had been delivered to Burma up to three years ago as part of a Unicef contract. Furthermore, he claims that, at the time of purchase by the institute's representative, the vaccine was three months past the printed expiry date — with potentially lethal consequences for anyone to whom it was administered.

Investigation by in the institute's laboratories in Switzerland revealed that the vaccine samples had lost their original colour and texture. Cryz says that despite vigorous shaking, the vaccines were extremely difficult to draw into a syringe, while the tetanus vaccine, which had been stored in unrefrigerated conditions, had lost potency and was toxic when injected in laboratory animals.

"This is very depressing," Cryz says. He points out that not only are Unicef vaccines apparently being diverted for economic gain, but many children "undoubtedly remain vulnerable to life-threatening diseases due to this practice".

Cryz adds that unless Unicef clamps down on the black-market diversion of vaccine supplies, manufacturers such as the Swiss Serum and Vaccine Institute will be reluctant to provide vaccines at cost price "while others profit at the expense of those who need safe and effective vaccines". He suspects the practice is not limited to Burma, and is attempting to confirm reports of similar activities involving two other South-East Asian countries, thought to be Cambodia and Laos.

Ehsan Masood

NIH drops reasonable pricing clause

Washington. Harold Varmus, the director of the National Institutes of Health (NIH), last week made the widely-anticipated announcement that NIH is planning to drop the unpopular 'reasonable pricing' clause from its cooperative research agreements with industry.

The clause was inserted into the agreements that cover cooperative projects between industry and intramural scientists in 1989 following outrage in Congress and elsewhere at the price being charged for the AIDS drug AZT, even though this had been partly developed with federal funds.

That clause effectively gave the government a right to decide the price of drugs developed in any cooperative project with industry, irrespective of how great or small industry's contribution was to the research.

But many companies have since refused to enter cooperative research with intramural researchers, arguing that the existence of the reasonable pricing clause, and the prospect of government involvement in price setting, has discouraged investors.

Over the past year, intramural researchers at NIH have added their voice to the industrial chorus calling for removal of the clause, on the grounds that it has become an impediment to arranging private sector support for their work.

In an attempt to resolve the conflicts that have arisen, Varmus has held two public meetings on the reasonable pricing clause,

as well as extensive discussions with the Department of Health and Human Services.

The NIH has now concluded that the cooperative research agreements, despite constituting a small fraction of NIH's intramural activities, are still an important way of advancing biomedical research by encouraging exchange of experimental compounds. To remove the barrier to forming such agreements, NIH is dropping the clause.

Not all groups wanted to see the clause dropped. David Barr, director of treatment, education and advocacy of the Gay Men's Health Crisis in New York, described industry's reluctance to enter research agreements because of the reasonable pricing clause as "slightly hysterical", given the lack of any example of such a clause being abused. "It is all based on how it might be used, and not how it actually was used."

In a statement, the NIH says it acknowledges the existence of such concern. But it points out that AZT, the drug that instigated the clause, was not developed under a formal cooperative research and development agreement (CRADA).

Varmus himself says that the price at which a drug is marketed should not be the responsibility of the NIH, even if its scientists have contributed to its discovery and development. "The NIH's primary programmatic mission, legislative mandate and expertise is in biomedical research, not in product pricing," he says. **Helen Gavaghan**

Research involving animals

SIR — The strength of opinion on both sides of the debate about the use of animals in research has generated mutual suspicion and discouraged rational discussion. In an attempt to break through the distrust, a group of antivivisectionists, members of animal welfare, research support and medical organizations, veterinarians, scientists using animals, members of bodies funding or directly engaged in research, moral philosophers and others, has been meeting over the past two years to exchange views. The participants were invited on the basis of their interest, knowledge and concern rather than as formal representatives of their organizations.

The discussions have been frank and wide-ranging, but constructive. Many of us were surprised by the extent of agreement on important issues, including commitment to the reduction, refinement and replacement of animals in research wherever possible, and unanimous condemnation of violence and intimidation against individuals and institutions.

We identified a number of areas for productive discussion, mainly centred on questions of openness and accountability, and especially the 'cost-benefit' analysis that forms part of the review of applications for project licences required for each programme of research under UK law. This essentially ethical judgement is ultimately in the hands of the Home Office, advised by its Animal Procedures Committee.

The group appointed a subcommittee to consider how accountability might be improved. The subcommittee focused on the scepticism engendered by the lack of openness in ethical assessment in the United Kingdom compared with others in which mandatory local institutional committees review proposals for research involving animals.

A discussion document prepared by the subcommittee identifies a number of potential advantages (to both sides of this debate) in the operation of institutional ethics committees:

- opportunity for discussion of ethical issues in animal research, particularly in the local context, thus encouraging best practice and assisting the essential cost-benefit analysis;
- widening consultation on animal research issues, improving the soundness of, and confidence in, decisions in this area;
- helping to create an environment in which broader educational benefits might follow within the institution;
- serving as an authoritative source of information about work in the institution, helping to protect individual researchers from harassment or misrepresentation of their work.

Potential disadvantages are also recognized:

- possible contradiction or duplication of the obligatory role of the Home Office;
- non-uniformity of policy or practice on animal issues across the country;
- increase in bureaucracy and cost in the regulation of research;
- the possibility that the involvement of more people in the ethical review might unreasonably delay or restrict research and even compromise confidentiality and the safety of researchers.

Subsequent discussion in the larger group concentrated on concern about the nature and role of 'lay' members, possibly including representatives of animal welfare/rights organizations, and the relationship between local committees and the statutory function of the Home Office. The latter problem might be dealt with, without changing the law, by making local committees advisory to the holder of the institution's Certificate of Designation, who must sign all project licence applications.

The group would welcome communications from organizations and individuals with experience of the operation of local ethics committees or who have views on this subject, particularly concerning the necessity for and role of lay representation.

Correspondence and requests for copies of the discussion document should be addressed to: The Boyd Group, PO Box 12421, Edinburgh EH2 4YB.

The positive and workmanlike approach of members of this group, despite their diverse backgrounds and positions, makes us hopeful that it may be possible to replace the kind of polarized argument that has characterized the debate on animal experimentation with a serious and constructive exchange of views.

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Apoptosis

SIR — John Funder (*Nature* 371, 98; 1994) was correct to call for pronouncing the second p in apoptosis, if we are to judge from words with related structures. The closest analogue is proptosis, which, like apoptosis, has a Greek prefix ending in o before the word ptosis, with main stress on

the long vowel o.

Searching on the electronic version of the *American Heritage Dictionary III* (1994 version), we found several dozen words (plus words derived from them) in which a form in -o is prefixed to a root beginning with pt. Without exception, this source calls for syllabifying the p of pt with the preceding syllable and pronouncing it there. Examples: acanthopterygian, archaeopteryx, coleoptile, haemoptysis, Neoptolemus, stearoptene. Similarly for words with a Greek prefix in -i — dipterous, triptych, peripteral — the p of pt is pronounced.

We do not have access to other electronic dictionaries, but we scanned Dorland's and Stedman's medical dictionaries plus *Webster's New International Dictionary* (3rd edition, 1976), and the *Oxford English Dictionary* (Second edition, 1984), which all agree with the pronunciations given in the *American Heritage Dictionary*.

Nonetheless, the confusion reflected in your correspondence columns extends to the experts. Dorland's and Stedman's disagree on the pronunciation of apoptosis. Dorland's opts for the p, while Stedman's leaves it out, though both sound the p in proptosis. But the analogues listed above make the pattern clear: the p of pt is not pronounced at the beginnings of words but is pronounced when it can be attached to a preceding syllable.

Søren Nørby (*Nature* 372, 312; 1994) calls for silencing the p of apoptosis in order to secure the understanding of the underlying science. We wonder whether he would agree to stop shifting the stress of atom in the word atomic for the same reason. In the accepted pronunciation of atomic, both of the vowels of atom are radically changed. We should also note that his example aminopterin, which he proposes as having a silent p, is incorrect, as in all the above-mentioned dictionaries the p is pronounced. Jonathan C. Busser and Alexandra Horowitz (*Nature* 372, 312; 1994) call attention to the example neuropsychology, which, like the base word psychology, does not get its p pronounced. But this is not revealing. Roots beginning with ps, such as psych, follow a different rule than roots that start with pt. The p is silent not only at the beginning of words but also following any prefixes. Thus, they are unlike pto, or pter, which, as we have seen, regularly retain the p after a vowel prefix.

On the basis of our search of the above-mentioned dictionaries, we conclude that the second p is not silent in apoptosis.

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Misunderstanding about COPUS

SIR — Your leading article about public understanding of science (*Nature* 374, 291–292; 1995) is misleading in relation to COPUS.

COPUS (the Committee on the Public Understanding of Science of the Royal Society, British Association for the Advancement of Science and Royal Institution) was set up against a backdrop of considerable apathy and sometimes resistance from the scientific and other communities involved in science communication. It was set very general objectives by its founding bodies, perhaps modest but only with hindsight. This generality enabled COPUS to develop a broad programme of its own quite closely directed activities aimed at clearly defined and diverse target groups such as scientists and engineers themselves, government and civil servants, media, publishers, women and others. COPUS has also funded hundreds of other grassroots things and actively encouraged other bodies to do more too.

Over the ten years of COPUS's existence, many other bodies have also contributed greatly. What the White Paper did was to build on that effort and take it far beyond what one committee could ever have achieved on its own. Since then, the OST has continued to be active in pursuing its public understanding of science mission.

The aim of COPUS was never to stimulate the support of voters for basic research but to encourage the public's interest in science and to help people to take part in discussion on issues that affect their lives. A major goal has been to encourage scientists themselves to be more open and communicative.

Public understanding of science is not simple or someone would have cracked it by now. COPUS does have its limitations. We are well aware of this and commissioned an external evaluation of the programme to help us decide our future role. The report is just complete and COPUS will be responding over the next few weeks. Certainly COPUS needs to involve many more people in its discussions — people who also need to know more about each other. A looser, more inclusive model must emerge that helps the debate of these important issues to develop in the United Kingdom. We have come a long way in raising public understanding of science as a matter for serious debate.

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Coloured slides

SIR — There seem to me to be several good reasons for using blue projection slides (white letters on blue background) rather than black and white¹. (1) Production is simple because, having made a negative, several blue copies can be made on different occasions using a simple ultraviolet (UV) light box and development in ammonia vapour (this may be necessary as they do fade with use), rather than wet-copying of negatives or contrast-reversal development of the original photographic film; (2) the intensity of blue, and hence to some extent the contrast, can be varied by length of UV exposure, being inversely related to exposure time; (3) if many slides are shown, blue and white is less tiring than black-white or white-black; and (4) they can be seen at low levels of ambient lighting while allowing enough light to take notes by. This last would of course also be true for black-on-white slides.

I have asked a number of colleagues, and the majority say they find blue a 'restful' colour, relaxing but not soporific; the manufacturers make similar sheet film that develops green, brown or red backgrounds, but these colours are not perceived as having the same authoritative calm as blue. I see no reason why the standards of the advertising industry should be applied to the exchange of scientific information, and do not believe that the increasing use of black text on white background on the computer screen has anything to do with the effectiveness of projection slides.

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SIR — Steiner Øvrebø raises an interesting hare¹. He tries to establish the preference of readers for black text on white background — against white on blue or diazo slides and computer screens. He cites research by IBM to corroborate his opinion. In reality, the truth is probably the reverse of what he claims.

By far the most common experimental finding in published books and journals is that silent reading from a screen is slower than reading from paper^{2–6}. The evidence is compelling and well documented. The doubt is in terms of the different experimental conditions. It is not clear, as Dillon⁷ points out, whether there is a generalizable effect, as subjects in the independent experiments referred to were treated with different stimulus materials. For example, Muter³ used white text on blue, whereas Gould and Grischkowsky⁵ used green text on a dark background.

Although the evidence refutes the IBM research referred to, there are other factors that may affect the choice of screen colour and screen fonts. It may be that

silent reading is faster on paper, but one would have to know the answers to a number of questions. First, was the accuracy of reading affected? Did subjects show or report any fatigue? How did comprehension scores change over different treatments? Did subjects or readers express any subjective or aesthetic preference for one colour system over another?

Øvrebø makes an interesting point, but such "commonsense theorizing" needs more rigorous examination. It is not sufficient to claim that "blue slides reduce readability and comprehension by about 20 per cent" without some backing and reproducible experimental findings.

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Adaptive events

SIR — I take issue with John Godfrey's reasoning (*Nature* 373, 100; 1995) that "during this period [of fertilization, that takes about two days] genetic identity is yet to be established", and that it does not start with activation of egg, a process that "precedes the chromosomal events that establish the genetic identity of the zygote". It is unlikely that this process can start and evolve without any role played by that specific egg and that specific sperm. The evolution of the process will be determined by a variety of events and conditions, but the start of the process, that will result in an individual life, precedes the process itself and its marvellous complexity.

Godfrey claims that "even after the fusion of gametes stable individuality is some way off". But, when will stable individuality really be established during the process of human life? Every stage after birth is a series of continuous adaptive events, in which every cell in human tissues participates. Human individuality also derives from the function of nervous circuits that are changed by internal and external events. Thus, a stable individuality could never exist and no man or woman would be a stable individual person with rights and duties.

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How to help Russian science

SIR — Pity for Russian science has become fashionable, as well as fear of the 'new Russians'. But there is also a new class of 'new paupers' in Russia — a large group that includes scientists. The rights of scientists have been betrayed by society, by the government, by those wanting to promote capitalism and by bourgeois interests.

The struggle for survival has become an everyday reality for scientists. Salaries range from the equivalent of US\$30 a month for an MSc to \$100 a month for a professor, while the cost of living is approaching that elsewhere. Transformation of Russia from a superpower to a developing country also means that the number of scientists may be reduced to only a tenth of what it was.

Russian science is threatened with extinction. Experienced scientists are either emigrating or moving into business in pursuit of higher salaries. Fundamental science has always been one of Russia's strengths but now commercialization is taking over.

Large research institutes belonging to the different academies of science and covering all fields of study are desperately struggling to survive stringent budget cuts. They often occupy huge buildings and have inflexible administrative structures. Hopes even for the money allocated in the 1995 budget are disappearing with every day of war in dissident republics. Many research institutes delay the payment of salaries and scientists wait to be laid off. International programmes, however generous, cannot solve the problems of Russian science and help only to prolong the agony. Newly established foundations such as the Russian Foundation for Fundamental Research cannot support even a limited number of research groups for lack of funds.

Thousands of talented young scientists between the ages of 20 and 30 have joined the 'brain drain' to the West. Older scientists with both theoretical and practical experience continue to work in Russia: they are the people most in need of help.

At the national level, I have the following proposals:

- Research centres consisting of active groups with joint scientific programmes should be organized to solve the acute scientific problems.

- An independent trade union should be set up to help scientists to find jobs and to help those already in work to establish contacts with potential customers.

- Laboratory space and equipment should be provided to such research groups.

At an international level:

- Laboratories elsewhere should provide opportunities for active Russian scientists

to visit.

- Western scientific organizations and companies could cooperate directly with active Russian groups in certain active areas.

- An organization should be set up to develop science strategies and projects.

- English-speaking universities should be set up in cities such as Moscow and St Petersburg and should employ Russian scientists.

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Popov, Marconi and radio

SIR — The centenary this year of wireless communication, or radio, brings to mind Professor Alexandr S. Popov (1859–1906), who together with Heinrich R. Hertz laid the foundation stone of the discovery. Hertz created the source of electromagnetic waves, then called 'hertzian waves'; Popov developed the first receiver of electromagnetic waves. Augusto Righi¹ wrote: "The new features of Popov's apparatus are the use of an electrically moved hammer or electric bell, to restore the initial resistance of the coherer, and the use of a vertical conductor, called later antenna, for receiving the waves."

The first demonstration of the hertzian wave receiver as 'a lightning detector' took place at the St Petersburg Physical Society on 7 May 1895 (refs 2, 3), and Popov demonstrated the transmission of intelligent signals in March 1896 to an audience of scientists from the society⁴. Popov was at that time teaching at the Russian Navy's Torpedo School in Kronstadt^{5,6}, and open publication about the transmission of intelligent signals was restricted by the Navy⁷.

Popov continued to investigate electromagnetic wave propagation and worked on the development of the first wireless communication systems in Russia. But production of wireless telegraphy was apparatus not successful in Russia. On the eve of the Russo-Japanese War of 1904–05, the Russian Navy was forced to use German-made radio equipment^{6,7}.

Popov was a physicist and an electrical engineer with wide interests. In 1901, he became a professor at the St Petersburg Institute of Electrical Engineering, and in 1905 was elected director (rector). In December 1905 he was ordered by governor of St Petersburg to take repressive measures against student political disturbances. He refused. His health was badly

affected and he died soon afterwards⁸.

Who could be said to be the inventor of radio, Popov or Marconi? The best answer is given by the historian Mouromtseff: "It is hardly possible to establish with certainty on which particular day either inventor conceived his final idea of wireless communication. Is it, however, necessary? From all evidence it is clear that Russia learned about wireless and obtained it in practical form through the knowledge and ingenuity of the scientist Professor Popov; the Western world unquestionably received all that through the energy and ingenuity of the young Marconi, and his unabatable faith in the future of radio"⁹.

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Bigger still

SIR — Henry Miller (*Nature* **373**, 652; 1995) is irked that H. C. Bennett-Clark states in his review of *Diatoms to Dinosaurs* (*Nature* **372**, 629; 1994) that: "Living organisms range in size from a diameter of about one hundredth that of a typical human cell to a length of 30 metres, with weights ranging from the infinitesimal to that of a medium-sized airliner". Miller remarks that: "Perhaps because Bennett-Clark is a zoologist he has overlooked the fact that there is another kingdom of living things called plants, which include the largest of all living things, the sequoia trees of California, estimated to weigh more than 1,000 tons."

Perhaps because Miller is a plant chauvinist he has overlooked yet another kingdom of living things called fungi. A thallus of the fungus *Armillaria bulbosa* was reported to have spread over some 15 hectares in soil in the forests of North America, is estimated to be more than 10,000 kg in weight and to be some 1,500 years old (*Nature* **356**, 382 & **356**, 428; 1992)! To keep the facts straight, much of the mass of the sequoia tree is contributed by the dead xylem cells.

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Molecular medicine in development

The case for modernizing the practice of medicine in the developing countries of the world is strong and also irresistible, but the mechanisms for doing that need to be improved.

Oxford. What can rich countries do to improve the quality of biomedical science in the rest of the world? That question occupied an absorbing two-day meeting in Oxford last week at which a score of academics from developing and (mostly) semi-developed countries set out their shopping lists. The gathering was organized by the Oxford International Biomedical Centre, intended as a kind of intellectual brokerage and which is the creation of cell biologist Professor Charles Pasternak of St George's Medical School in London. (The Oxford connection consists chiefly of Pasternak's wish eventually to erect a building on a site he has identified.)

What follows is not so much a report of the proceedings as an account of some of the issues that arose, often implicitly, from what people said about the problems of teaching medicine and delivering health care in countries such as China, India, Kenya, Poland and those of Latin America.

One central issue is the extent to which the medical curriculum, and the pattern of research, should take account of the past few decades of understanding gathered by molecular biologists. Lu Shen Dong, a vice-president of the Chinese Academy of Medical Sciences, asserted that gene therapy now stands alongside traditional Chinese medicine in the country's 131 medical schools. Most other participants were alarmed that their systems would be deprived of therapeutic opportunities by the concentration of modern biology in the rich countries of the world.

But why invest money and energy in learning the most up-to-date techniques when the principal causes of death and disability can be traced to old-fashioned causes, preventable infections and public hygiene? The question echoes the arguments in the 1960s about India's wisdom in investing expensively in the development of nuclear energy, when the country's economic and social problems had more familiar causes. (India may not have benefited much from nuclear energy as such, but the recent success of its chemical and electronic industries are identifiably by-products of the original development.)

Last week's gathering was in no doubt that modern techniques in biology must find their way into medicine everywhere, and quickly. For one thing, new techniques may help shed present burdens of morbidity, as hepatitis-B vaccine may reduce the incidence of liver cancer. But the general case for including modern medicine in the

curriculum is the career life expectancy of physicians, who will be working with patients for decades ahead. According to Professor Maciej Nalecz, director of the Nencki Institute of Experimental Biology in Warsaw, one of Polish medicine's most urgent needs is to retrain the physicians already at work in hospitals.

Nalecz also had a cheerful tale to tell about the establishment in Warsaw of an International Centre for Cell and Molecular Biology, jointly financed by UNESCO and the Polish government, and due to open this October. He regards this both as a means by which professionals at all levels can be given some training in molecular medicine and a way of drawing other Central European countries into collaborative research programmes.

In some respects, the new centre is modelled on the European Molecular Biology Laboratory in Heidelberg, but there will be no hard core of permanent staff; instead, researchers will be employed on three-year fellowships. Management will be by a steering committee on which participating countries will be represented. If the new centre makes a good start, there is every reason for Nalecz's belief that Poland has a model research centre that might meet some of the needs of other regions of the world, Latin America for example.

Curious as it may seem, Slovenia (represented by Professor Vito Turk of the J. Stefan Institute at Ljubljana), only recently the most northern part of Yugoslavia, is more self-confident, perhaps because its economy rests on substantial pharmaceutical and electronic industries (as well as on agriculture). The plea by Professor Arnost Kotyk, from the Institute of Physiology at Prague, that Central Europe should worry about the poor salaries paid to people working in research was widely welcomed, not simply by those from the region.

That is one of the well-springs of another insistent theme last week: the steady loss of trained people from developing and semideveloped countries. Lu, for example, said that of 2,000 physicians sent for specialist training overseas by China in the past 10 years, only 1,000 had so far returned. Dr Rem Petrov, an immunologist who is also a vice-president of the Russian Academy of Sciences, appeared more sanguine about the loss of people from Russian institutes, estimating it at about 10 per cent by emigration (and rather more by the loss of people to other activities); he did not remark on the circumstance that the

brightest are the first to leave.

So what is to be done about the 'brain drain'? One speaker held that it is "immoral" for rich countries to recruit poor countries' skill in the deliberate way in which (some alleged) they do. The counter view is that it would be an intolerable intrusion on the personal liberty of researchers that they should be prevented from moving countries, but that it is only prudent that governments looking for further training for their specialists elsewhere should prudently skew the terms in such a way that their people eventually return. That, of course, is one respect in which Kotyk's opinion about the pay of researchers will in the end be telling.

The problems of the truly poor countries were not much advertised last week; they were not represented. But India (which is two countries, one rich and one poor, with the former growing quickly) produces 17,000 qualified physicians a year, but still cannot provide for the health needs of the rural poor. It is the same in South Africa: Dr Chris Hugo-Hamman, a member of the South African delegation to the European Union, said that there are 1,200 people for every physician in the white communities, but 12,000 to one in the black communities. The "crisis" in South Africa's education, he said, is that only 1 out of 316 black students finds his or her way to a university.

Kenya seems even worse off. Joseph Ruto, the high commissioner for Kenya in London, said that there are insufficient funds for health care and for running the medical schools and that the country is once again assaulted by malaria and tuberculosis as well as by HIV. Dr Baldip Khan of the Kenyan Medical Research Centre at Kenri, whose own field is the search for antigens effective in developing vaccines against schistosomiasis, told a moving tale of the sense of isolation she shares with 39 colleagues and of the encumbrance of having to walk for half an hour to read immunoassays in another building.

What is the rest of the world doing to help? The UNESCO centre in Warsaw is something to boast about. Otherwise, there is a plethora of fellowship and scholarship schemes. The Oxford centre is pushing for a standardized one-year fellowship scheme for medically qualified people. An equally important need is some way of simplifying and making uniform the schemes that exist already, many of which appear to be under-used, probably for lack of information.

John Maddox

Principle of least astonishment

Ronald T. Merrill

THREE papers published a decade ago¹⁻³ suggested that exceptionally rapid changes in the Earth's main magnetic field were recorded in two 16-million-year-old lava flows at Steens Mountain in Oregon, USA. The changes were orders of magnitude faster than seen in direct measurements of the field, and it was widely believed that even if there were such rapid changes in that part of the magnetic field originating in the Earth's core, they should be screened out by the overlying 2,900-km-thick semiconducting mantle. So most geomagnetists dismissed the claim by applying the principle of least astonishment: it was easier to believe that these lava flows did not accurately record the changes in Earth's magnetic field than to believe that there was something fundamentally wrong with the conventional wisdom of the day. And there the story might have ended, except that two of the scientists involved in the original work, Coe and Prévot, insisted on keeping the subject alive. They are joined by Camps on page 687 of this issue⁴ to argue that the changes recorded at Steens Mountain do reflect changes in the Earth's main field, and that such changes sometimes occurred at twice the rate originally reported — up to 6° per day, the authors now say.

Field origin

The 'main' magnetic field is believed to originate from electric currents in the Earth's outer core. Seismic and mineral physics results indicate that the outer core primarily consists of liquid iron. Although this implies that the outer core is a good electrical conductor, estimates of ohmic dissipation indicate that currents in the core should still die away on a timescale of the order of 10⁴ years if there is no new energy input to sustain them. The longevity of the Earth's magnetic field (it has existed for at least 3 billion years) indicates there must be some such energy source, most probably mechanical energy associated with fluid convection in the outer core. There are many dynamo models that attempt to give the details of how this mechanical energy is converted to electromagnetic energy, but because not all the critical parameters for the Earth's interior are known well enough and because of difficulties associated with the mathematics, none is fully adequate. Thus much of our knowledge of the Earth's field comes from direct surface measurements over the past few hundred years and from indirect measurements of its history as recorded in rocks⁵.

The direct measurements indicate that the field changes only slowly, so that, for instance, magnetic compasses are useful

for navigation. This would not be the case at a time when the field was changing at an average rate of 6° per day over several days, as Coe *et al.*⁴ now suggest. They claim that such changes occurred when the Earth's magnetic field was changing polarity: the geomagnetic north and south poles were trading hemispheres.

Hundreds of such magnetic field reversals are now well documented. In general it takes about 5,000 years for a total reversal to occur; during this time the field intensity markedly decreases but it never vanishes⁵. The authors agree with all this, but they also believe that on occasion during a reversal the magnetic field can undergo extremely rapid changes.

Their evidence comes from palaeomagnetism. When a lava flow cools, it typically acquires a magnetization parallel to the external magnetic field. This is referred to as a primary magnetization, and provided it does not change with time, one has a record of the Earth's field at the time the lava flow cooled.

The authors have found a few locations at Steens Mountain where there are large changes in the magnetic directions within a single lava flow (and when the field intensity was of moderate size). These lava flows are fairly thin, and, by assuming a conservative cooling history for the flows, Coe *et al.* estimate the rate at which the primary magnetization was acquired.

In previous work of this kind, critics pointed out that the magnetization might not be primary; it is not uncommon to find lava flows that have been remagnetized long after they cool, for example because of chemical alteration. So some critics concluded that the alleged rapid changes in the Earth's field really reflected an imperfection in the magnetic recording process. The authors tackle such criticism head-on in their new paper, and they make a convincing case against the 'magnetic artefact' argument. Similarly, they present a convincing case against other hypotheses, such as that the changes in the magnetization reflect changes in the external magnetic field associated with, say, a magnetic storm.

Coe *et al.* also argue that the data are compatible with the present electrical conductivity structure of the mantle, but this claim is less convincing. Their model for the mantle conductivity distribution is an insulating mantle to a depth of 660 km, below which the conductivity jumps to a value of 1 S m⁻¹ and retains this value to the boundary with the Earth's core. This is a reasonable distribution for modelling purposes, but it probably lies at the lower end of acceptable models. For example, the lowermost mantle may contain regions

of conductivity orders of magnitude higher than the values in this model⁶. Indeed, a much higher conductivity in moderately large regions seems required if electromagnetic coupling between the core and mantle significantly affects the observed decadal variations in the length of day⁷.

Filtering

An apparent contradiction yet to be resolved is that statistical analyses^{8,9} of variations in the Earth's magnetic field indicate that the mantle acts like a filter by sharply reducing signals with periods less than about a year. In contrast, Coe *et al.* say they have used the conductivity distribution given above and a well-respected theory developed by George Backus to calculate that only variations in the dipole field with periods less than 20 days would be screened out by the mantle.

Even so, this is still inadequate, given the high rate of change they observe. To circumvent this problem, they calculate that a magnetic field characterized by a spherical harmonic of degree 6 would not be effectively screened out until it had a period of 6 days, a value that is just compatible with their data. However, the amplitude of such a field would be about two orders of magnitude smaller at the Earth's surface than at the core-mantle boundary and moreover, it would have to be substantially larger than fields characterized by lower-degree harmonics. This would be a very unusual field for the intensities they quote (any short-period variation in magnetic field will get through the mantle if it has a large enough initial amplitude). In short, if Coe *et al.* are correct, then the consequences could be much more profound than they say.

All this leaves us with a dilemma: we would like to apply the principle of least astonishment, but to which data and interpretations? Some scientists will accept the view as given by the authors. Others, I suspect, will choose to believe the rock magnetic record is still inaccurate; unquestionably the work of Coe *et al.* makes the artefact hypothesis a contrived one, but it cannot eliminate it. Still others might apply the same principle to argue that our picture of mantle conductivity is substantially in error.

There is yet another possibility. The

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mantle filtering theory used in these analyses assumes that the conductivity varies only in the radial direction. Yet magnetotelluric and other geomagnetic induction studies have shown that there are significant three-dimensional variations in electrical conductivity in the upper mantle. The magnetic field diffuses much more slowly through a good electrical conductor than a poor one. Could such structures distort an initially slowly varying field of core origin in such a way that changes at the surface occur much

more rapidly? Calculations will be necessary to see if this speculation has any substance.

Regardless of one's point of view, one can but admire the care with which Coe *et al.* have done their experimental work. Eventually, the consequences should be profound. □

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DEVELOPMENTAL NEUROBIOLOGY

A real gene for *reeler*

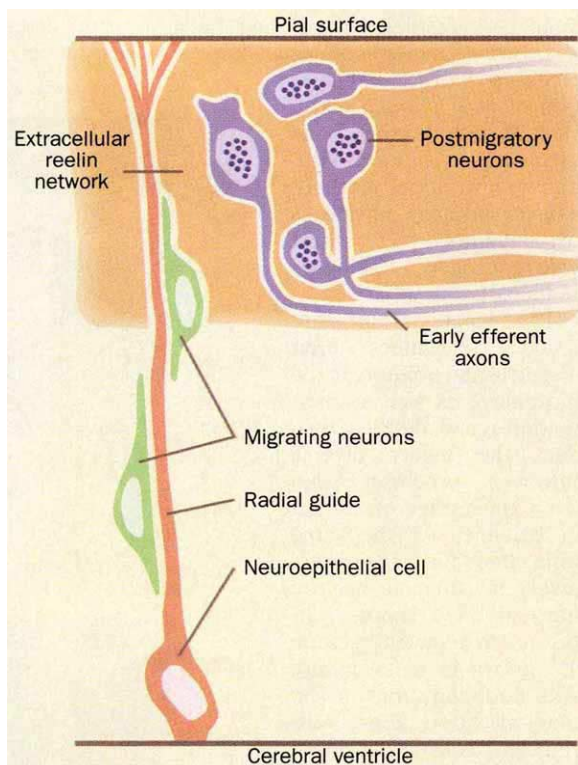
André M. Goffinet

EVERY so often a paper comes along which shows we are indeed making significant progress towards understanding the sheer complexity of the mammalian brain, with its billions of neurons and connections, and how it develops in the embryo. One such appears on page 719 of this issue¹, where D'Arcangelo and colleagues report the cloning of the mouse *reeler* gene. It is a gem of a paper — although the authors say that the evidence is not entirely definitive, I find it compelling.

The *reeler* mutation has puzzled developmental neurobiologists for nearly 50 years, the long-lasting interest stemming from the unique phenotype that results (reviewed in refs 2 and 3). Briefly, in homozygous *reeler* embryos, neurons are generated in normal numbers, at the normal time. Initial neuronal migration occurs normally, as does differentiation of the various cell types, axonal and dendritic deployment, and formation of connections. However, from embryonic day 13–14, when the first migrating neurons reach their destination, cell patterning goes awry. This selective architectonic defect is most obvious in the cerebellar and cerebral cortices, but it occurs almost everywhere in the central nervous system.

Although the *reeler* mouse has been used successfully as a model of abnormal corticogenesis, the mutation's mechanism of action has remained unknown. Some investigators favoured the idea that aberrant *reeler* protein dis-

turbs cell interactions between radial glial cells and migrating neurons⁴, whereas others, including myself, thought it more likely that it affects homotypic adhesion between early postmigratory neurons^{2,3}. Interestingly, Derer and Nakanishi⁵ have proposed that extracellular, matrix-mediated cell interactions are disrupted by the defect. No satisfactory experimental approach could be found to progress along any of those lines, and the field has been relatively stagnant for the past few years.



A model for reelin action in cortical development. Neurons are generated in ventricular zones, along the cerebral ventricles, and migrate radially along the extensions of neuroepithelial (later glial) cells. Postmigratory neurons express the *reelin* gene as part of their differentiation programme. Reelin protein is incorporated in the extracellular matrix where it helps to stabilize cortical architectonic patterns.

Now *reeler* is back with a vengeance, with the appearance of the paper in this issue and the impending publication of three others. D'Arcangelo *et al.*¹ report the full-length *reeler* complementary DNA and present a first analysis of the deduced protein, dubbed *reelin*. Two other studies using positional cloning strategies^{6,7} provide incomplete sequence data that confirm the more complete work¹ (although the length of the messenger RNA and size of the protein deduced by Hirotsune *et al.*⁷ turn out to be wrong). A fourth paper⁸ describes the characterization of an epitope that is specifically absent in *reeler* mice.

The results of D'Arcangelo *et al.* are highly impressive. The *reeler* gene encodes a long mRNA of about 12 kilobases. *Reelin* is a huge protein of 3,461 residues (relative molecular mass, 388K), with features that are highly indicative of a secreted extracellular protein. It shows homology to F-spondin, a protein secreted by the floor plate of the developing spinal cord that may contribute to the growth and guidance of axons in both the spinal cord and the peripheral nervous system⁹, and to tenascin, a large, hexameric, extracellular matrix protein¹⁰.

The *reelin* RNA is first detected in the embryonic brain on day 11.5. It then increases in concentration up to birth, remains high during the postnatal period and then declines progressively to adult levels; it is brain-specific. *In situ* hybridization studies detect the *reelin* RNA in postmigratory neurons in the external zone of the hemisphere at embryonic day 13.5, just before the *reeler* phenotype can be detected. The *reelin* transcript is also seen in early postmigratory neurons in other telencephalic fields. In external granule cells of the cerebellum, expression seems to be initiated at the end of tangential migration. Most strikingly, no *reelin* expression is found in the ventricular zones where the cell bodies of radial neuroepithelial cells are located. At the level of the cerebral cortex, early expressing cells could correspond to Cajal–Retzius cells, transient neurons that may act as ‘pathfinder’ neurons and be involved in the primordial organization of the cortex. It is worth noting that the epitope described by Ogawa *et al.*⁸ is apparently also specific to Cajal–Retzius cells.

These results suggest a simple model of matrix-mediated radial corticogenesis, shown in the figure, which complements radial guidance of migrating neurons by glial cells. *Reelin* would be secreted locally by early postmigratory neurons to act as a neuron–matrix adhesion molecule and help stabilize early architectonic cell patterns^{2,3}. The term ‘stabilization’ should not be taken in a restrictive, purely mechanical sense; indeed, for neuron–matrix–neuron interactions to influence

cell patterns, they must be relayed to the interior of the cell, to the cytoskeleton and signalling machineries^{11,12}. In accord with previous studies of chimaeric mice (see for example ref. 13), the structure of reelin implies that it functions outside cells. To act as an architectonic-stabilization factor, reelin could interact with a cell-surface receptor, although the available data certainly do not rule out homophilic binding. To risk an educated guess, a reelin receptor might be analogous to the receptor-type protein phosphatase- β , the extracellular domain of which is similar to chondroitin sulphate proteoglycan and is able to bind to tenascin¹⁴.

The new data provide a basis to address this matter and many others. The function of reelin in the developing brain can be analysed in structural and cell biological studies. If the expression of reelin is an integral part of early neuronal differentiation, how is it controlled, particularly at the transcriptional level? If reelin is indeed an adhesion protein necessary for architectonic development, what would happen in transgenic mice that overexpress it? From comparative embryological studies³, it seems that the *reeler* gene participated in cortical evolution in the lineage leading from stem reptiles to mammals, and the cloning of *reelin* provides the opportunity to pursue this idea further.

Finally, the *reelin* gene is present in humans and maps to region 22 on the long arm of chromosome 7 (ref. 1). As yet, no human brain disorder has been mapped to this interval, and it could be that the *reeler* malformation is lethal in human embryos. Nevertheless, the possible role of the gene in human brain malformations and polygenic diseases such as schizophrenia will undoubtedly come under scrutiny.

The news of the cloning of *reeler* will be widely applauded by the neurobiological community. But — as ever — much work remains to be done, and it will keep several postdocs busy for several years. □

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Life at the sea floor

Holger W. Jannasch

THE microbial world continues to deliver surprises, the latest being announced by Fossing *et al.* on page 713 of this issue¹. Seemingly defying their typical dependence on diffusion for their supply of nutrients, certain microorganisms grow in massive mats on the sea floor along the west coast of South America; such is their density that they wreak havoc with the local Peruvian and Chilean fisheries by

were discussed here at Woods Hole³, I am only too aware of how little further information about them has been obtained since then. *Thioploca* has been known about for some time but data on the mats, which occur for a stretch of 3,000 kilometres, have been fragmentary and posed more questions than answers. Fossing and colleagues have now pulled the pieces together.

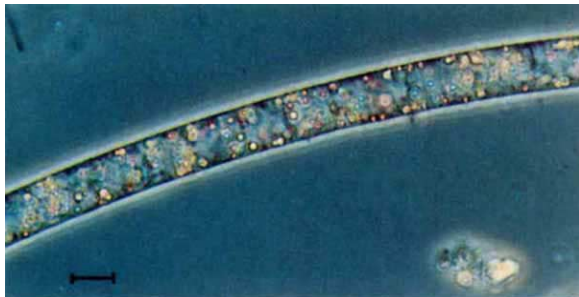


FIG. 1 Single *Thioploca* filament with enclosed sulphur globules. Scale bar, 40 μ m.

clogging up nets with what is known locally as *estopa* (the Spanish for uncleaned wool)². The principal constituent of these mats is a filamentous bacterium called *Thioploca* (Fig. 1), and its metabolic characteristics have long been a puzzle. Fossing and colleagues have now come up with the explanation of how *Thioploca* can exist at such densities, one involving a hitherto unknown nutritional mechanism for bringing nitrate as oxidant into direct contact with hydrogen sulphide as reductant.

The smallness of microbes guarantees their dispersal and ubiquity in the biosphere, as well as their readiness and ability to conduct the highly diverse turnover processes that keep the cycles of matter in balance. At the same time, their tiny size is necessary for them to acquire nutrients by simple diffusion. Whenever bacteria are grown in mass culture with doubling times of less than an hour, heavy stirring and constant nutrient supply are required. How, in principle, can things be different in nature?

Having been around at the time — 1977 — when the strange mats off the Peruvian and Chilean coast

In a nutshell, their results are these: identification of particular nutrient concentrations below a very rich upwelling zone, with unusually high levels of nitrate and near-zero oxygen; characterization of the ability of the *Thioploca* cells to accumulate nitrate to even higher concentrations within large liquid vacuoles, and of the highly efficient gliding mobility of the nitrate-laden cell

filaments within large slime sheaths; and elucidation of the unique metabolic role of this transport system, as depicted in Fig. 2a.

Thioploca, then, makes a living by bridging the nitrate-rich zone near the sediment surface, using nitrate as the

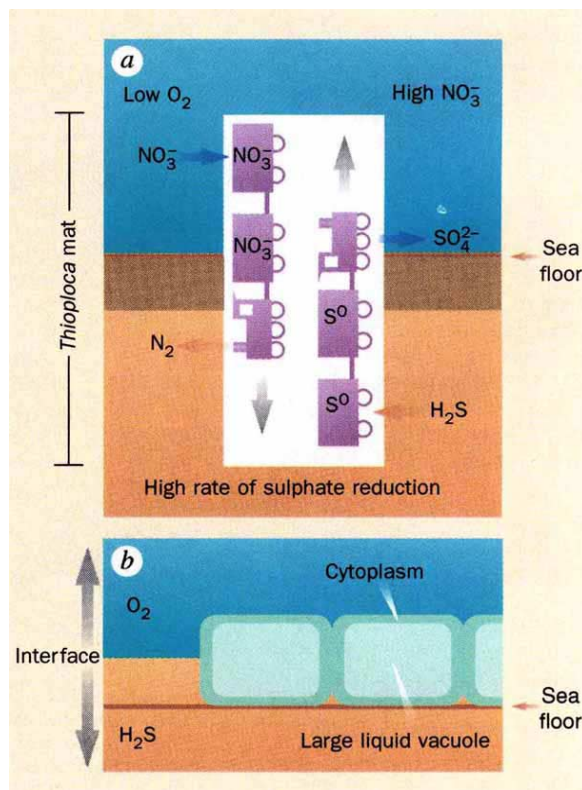


FIG. 2a, Stationary interface and moving *Thioploca* filaments. b, Moving interface and stationary *Beggiatoa* filaments.

electron acceptor, and the underlying zone containing hydrogen sulphide. Hydrogen sulphide is employed as the electron donor and globules of elemental sulphur, accumulating in the ascending cells, can be oxidized further to sulphate. This chemolitho-autotrophic way of life is complemented by heterotrophic bacterial sulphate reduction, resulting in the continuous delivery of hydrogen sulphide. The hydrogen sulphide is so efficiently taken up that it never reaches the sea floor, indicating that much of the filament movement must be vertical. This amazingly productive transport system overcomes the difficulty of garnering nutrients from two separate layers and allows *Thioploca* to grow in such unusually dense mats. The process is greatly helped by another unusual phenomenon: the large liquid vacuoles within the individual cells that can concentrate nitrate 20,000 times over the ambient concentration of 25 μM .

In these large vacuoles the diffusion problem is solved by the small amount and particular distribution of cytoplasm, which covers the inner cell wall in a thin layer and is thereby readily accessible to nutrients from the outside. This arrangement is similar to that in the even larger vacuoles of some filaments of *Beggiatoa*, a bacterium that is closely related to *Thioploca*. Massive mats of these *Beggiatoa* filaments occur at depths of 2,000 metres on the sea floor at sediment-covered vent sites where geothermally produced hydrogen sulphide emanates⁴. Here the sea water contains enough oxygen to serve *Beggiatoa*'s requirements for aerobic chemolithotrophism of hydrogen sulphide.

The striking difference from *Thioploca* is that the *Beggiatoa* filaments are usually stationary; instead, it is the interface between the two zones that moves⁵ (part *b* in Fig. 2). By loading up the large vacuoles with reducing or oxidizing materials, the cell is able to 'hold its breath' for a while until the interface moves again. In principle, the larger the intracellular space that can be filled during exposure to oxidizing conditions, the longer the cell can survive under reducing conditions, and vice versa. Although *Beggiatoa* filaments can also move by gliding, they never form mats thicker than 0.4 mm in the absence of a rising and falling interface⁶.

These are elegant solutions indeed for coping with a requirement for nutrients that do not occur in the same place or at the same time. Other implications apart,

the newly discovered transport system in the *Thioploca* mats is likely to have a pronounced effect on the nitrogen cycle in the South American coastal upwelling zone. That calls for investigation. Meantime, it is to the credit of the Max Planck Institute for Marine Microbiology at Bremen, Germany, which was founded only in 1992, that it took the initiative

MILKY WAY

A second superluminal source

Galen Gisler

HARD on the heels of the discovery of the first Galactic superluminal jet by Mirabel and Rodríguez¹, we now have a second such source. In contrast to the first, the hard X-ray transient GRS 1915+105, which has only been studied in detail at radio frequencies, this new source, variously known as X-ray nova Sco and GRO J1655-40, has been observed in the radio, in the optical and at high energies. Radio observations made by Tingay *et al.* were reported last month²; optical counterpart observations by Bailyn *et al.*³ and X-ray observations by Harmon *et al.*⁴ appear on pages 701 and 703 of this issue.

The high proper motion of this source's components, roughly 50 milliarcseconds per day, is absolutely unprecedented. Our species has long regarded the domains beyond the Solar System as immutable; 'fixed stars', as we used to say. Of course, we have come to know that the stars are not fixed to some crystalline sphere but that the Universe is dynamic, evolving and sometimes violent. Even so, an arcsecond per year has been thought of as huge for apparent motions against that crystalline sphere. Now we have apparent celestial motions more than an order of magnitude larger. This may not imply a grand shift in our thinking or in our cosmology, but it does present practical problems.

Very long baseline interferometry (VLBI) uses several radio telescopes spread across the Earth to increase the angular resolution far above that obtainable with single-dish radio telescopes. The rotation of the Earth changes the spatial relationships among the participating individual telescopes, as seen by the source being observed. The different spatial configurations at different times combine to sweep out a vast area, and so a conceptual telescope comparable to the size of the Earth itself is synthesized. The beam widths attainable with this technique can be as small as a few tens of milliarcseconds at the relevant frequencies, but the fundamental assumption of the usual algorithms is that the source being observed is stationary during the time required to synthesize the larger telescope. GRO J1655-40 violates this assumption.

and invested in a carefully planned research cruise to investigate *Thioploca* metabolism. As the paper by Fossing *et al.* attests, the effort has paid off handsomely. □

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So how does one do VLBI on a rapidly moving source? One approach is to chop the observational day into small enough segments that the fundamental assumption is valid within each segment; another is to use lower-resolution aperture synthesis methods (for instance, to observe the source with the Very Large Array) for a sanity check. Both these methods have been used so far in the study of this source. For the longer term, and in the hope of finding more extreme examples of this phenomenon, the development of an algorithm for resolving rapidly moving sources is desirable. There will surely be limits to such an algorithm, if indeed one is possible at all; one could never hope, for example, to resolve a very small source that traverses the entire visible sky during a 12-hour observation.

Of course the reason for the very large proper motion of this source is a combination of three things: a large intrinsic transverse velocity, the relativistic illusion (described in my earlier News and Views article⁵) that magnifies the transverse velocity, and the relative nearness of this particular source (just 3.5 kpc, according to the estimates). All of these qualities make GRO J1655-40 a highly interesting source, and one that may generate as much of the light and heat of scientific controversy as SS433⁶. That Galactic source, known since the late 1970s, has yielded much information on the dynamics of jets. The enormous radial velocity variations seen by optical spectroscopy, and the wiggly features seen in the radio images, led to a precessional corkscrew model that has pinned down quite accurately many of the parameters of the system, notably the jet velocity (0.26c).

The nearness of the new source has engendered hopes among astrophysicists that we may learn a great deal about the sort of violent activity that occurs in some of the most spectacular and exotic objects in the astrophysical gallery. Because there is an optical counterpart, the possibility of measuring radial velocities from spectral lines can, as in SS433, give us detailed information about the dynamics of the components of this source. Possibly this

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one will be just as hard to understand. Already there is a hint of a corkscrew-like wiggle in the jet structure, in data presented by R. M. Hjellming at the Tucson meeting of the American Astronomical Society in January⁷, but there is as yet no obvious optical periodicity corresponding to that wiggle.

Even more tantalizing is the observation reported by Baily *et al.*³ hinting of an optical eclipse in the source. Although this was not seen to repeat in their data, the potential leap in our understanding of this source that would result if an eclipse were present is enormous enough to justify a careful and thorough watch for future occurrences. Would that we were so lucky as to have a dark occulting body regularly pass in front of one of the most interesting objects in the sky!

In the case of the Sun, of course, eclipses taught us about the fascinating phenomena of prominences, flares, the corona and the solar magnetic field, long before we were able to observe these by other means. Eclipses in distant astronomical objects are likewise valuable for revealing what might be invisible at other times, or for associating some phenomenon with a particular spatial location. Pragmatically they are valuable because we can measure the times of eclipse start and end very accurately. From these times, and from the velocities that can (in principle) be obtained from spectroscopy, we can infer true spatial dimensions, and from the physics of gravity, true masses for the bodies involved. These physical quantities are essential to an understanding of the nature of the phenomenon. In the case of this source, such measurements could provide us with incontrovertible evidence for a black hole.

The universality of the jet phenomenon in energetic extragalactic sources, and its emergence as a relatively common phenomenon in energetic Galactic sources, truly begs for a deeper understanding of the physics involved in the generation of jets. While the stability and collimation of jets has been very thoroughly studied, particularly in the last decade and a half, we have made little progress on the understanding of how they begin, deep in the nuclei of these energetic systems. More detailed observations of objects like GRO J1655–40, GRS 1915+105 and SS433 can only help. □

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Seeking planets around nearby stars

Roger Angel and Adam Burrows

THE possibility of planets outside our Solar System has stirred people's imaginations for centuries. But scientists past and present have not been able to satisfy that curiosity with credible detections. Planets like those in our Solar System have been too dim and small to identify around even nearby stars. To be sure, Earth-mass objects are known from periodic Doppler shifts¹ to orbit one radio pulsar, but these must have been formed by more exotic means than the 'standard' planets that we seek. Today a variety of search strategies have matured to the point that they should find Jupiter-like planets if they are abundant. The sensitivity and time for detection with each technique depends differently on orbital radius. The question is, what is the expected distance between a star and its giant planets?

Jupiter zone

Enter Alan Boss of the Carnegie Institution in Washington DC. Boss provocatively concludes² that extrasolar giant planets are only likely to be formed within a narrow range of radii close to that of Jupiter (between 4.5 and 6 AU, where 1 AU is the distance from the Earth to the Sun) almost regardless of the mass of the central star, provided that lies in the range 0.1 to 1.0 solar masses ($M_{\odot}$). It is thought that giant planets such as Jupiter and Saturn, made predominantly of the hydrogen and helium of the protostellar disk, must nucleate around a core of ice and rock³. Inside the so-called ice condensation radius, the disk is too hot (over 160 K) for ices to condense into the planetesimals that seed the process and giant planets can not form, although the formation of terrestrial planets such as the Earth is not inhibited. Giant planets are expected to form not only outside but also near this critical radius⁴.

Using a radiation/hydrodynamics code, Boss assumes that the disk between 1 AU and 10 AU has a mass of $0.02 M_{\odot}$ near the start of giant planet formation, and that the spherical mass accretion rate onto the disk is 10^{-5} to $10^{-6} M_{\odot}$ per year. Then with the further assumption that there are no intrinsic heat sources in or around the disk, he calculates the steady-state disk temperature profile and hence the condensation radius for central stars with masses of 0.1, 0.5 and $1.0 M_{\odot}$. In this picture giant planets can form at only a narrow range of moderate distances from the central star, not nearby. If Boss is correct, what are the consequences for the different detection schemes?

The techniques currently in use to

search for planets are indirect: astrometry (looking for the wobble of the central star's position in response to the presence of a planet⁵), and spectroscopy (looking for periodic Doppler shifts in the frequencies of a star's absorption lines from the same motion⁶). If the heaviest planets giving the biggest wobble are typically at 5 AU radius, the orbital periods will all be long, from 12 years for a star like the Sun to 20 years for the much more common lower-mass stars. This means the reaction motion searches, which need data taken over a good fraction of an orbit for a sure detection, may not get any quick results, even though they are now reaching the required sensitivity. Not only would detection periods be long, but reaction velocities would be slow and correspondingly difficult to detect. We might search in vain for the strong Doppler signals caused by a fast-moving Jupiter-like planet orbiting close to a low-mass star.

Radii of 5 AU would be good news for the proposed detection of planets by gravitational lensing. A planet produces an aberration in the otherwise symmetrical lensing action of a star, causing an irregularity in the brightening of a background object⁷. This effect is maximized for orbital radii near the so-called Einstein radius, which for the searches now under way in the bulge of our Galaxy is thought to be a few AU, fortuitously near Boss's result. A sizeable separation between star and planet would also be advantageous if planets are to be discovered by direct imaging. Otherwise, overcoming the glare of the enormously brighter star close by is the challenge. Recent calculations show that given a 5-AU separation, new large telescopes on the ground corrected with adaptive optics should be capable of seeing planets out to 25 light years (ref. 8, and S. M. Stahl and D. G. Sandler, manuscript in preparation).

Prospects

With no planets yet detected from the ground, and no assurance that the largest and most readily detected planets will be common⁹, no space missions aimed at extrasolar planet research have been approved. But if a nearby extrasolar planet is discovered from the ground, the result could galvanize NASA. D. Goldin, NASA's administrator, has already put forward the exploration of planets beyond the Solar System as a visionary goal. The possibilities for exploration with space telescopes unhampered by atmospheric absorption and blurring are remarkable. Planets like the Earth would be detectable

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and, if found, measurements could be made of their chemical and physical characteristics as well as their masses and orbits. A space-based interferometer to detect the heat of a Jupiter-like planet was proposed by Bracewell in the 1970s¹⁰. Built on the scale of today's large ground-based telescopes, such a device could detect Earth-sized planets¹¹, and analyse their infrared spectra for atmospheric

water and oxygen, features that are, as far as we know, unique to the living Earth¹². Such a mission would challenge NASA to an extent unknown since the Apollo mission to the Moon. □

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cited into the upper states, there is a profound modification of the optical properties of the system due to the interference between the different possible excitation pathways.

One consequence of the interference is the vanishing, near resonance, of the absorption of light from the weak 'probe' beam (see Fig. 1). The frequency range over which modified absorption occurs is proportional to the electric field strength of the coupling laser. This effect, known as electromagnetically induced transparency (EIT)³, has been much researched in the past four years. An important consequence of EIT is the possibility of laser action in the absence of a population inversion⁴, because only absorption vanishes without there being a similar effect on stimulated emission.

Accompanying these modifications to absorption there will also be changes to the refraction (Fig. 2), as Scully⁵ pointed out in 1991. In particular he realized that, as a further consequence of the interference effects, it is possible to induce an anomalously large refractive index exactly where there is an almost vanishing absorption, so a medium is created in which there is a large refractive index but low loss. This is contrary to the normal situation in which the refractive index will only be large where the absorptive losses too are very large. The new experiments^{1,2} are concerned specifically with this refractive index enhancement.

Normally, to observe changes in the optical response of a gas or vapour it is necessary to use a laser with an electric field strength large enough to modify the absorption and refractive index over a frequency range exceeding the Doppler width of the transitions. For continuous-wave lasers, which were used in these experiments, the laser electric fields are

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OPTICS

Focusing light with light

Jon Marangos

TWO papers in a recent issue of *Physical Review Letters*^{1,2} demonstrate the subtleties now possible in using lasers to control the optical properties of a gas. Both deal with changes induced in the refractive properties of rubidium vapour, essentially an enhancement in the refractive index, caused by an intense resonant laser field. One of the groups (R. R. Moseley *et al.*² of the University of St Andrews) takes this a stage further by demonstrating laser-induced gas lenses whose sign and focal power can be varied by altering the intensity and tuning of the applied laser. This kind of laser control of optical properties could have considerable technological potential.

The basis of the effect is interference between alternative transition pathways in a quantum mechanical process, a well-known effect in physics which is analogous to constructive and destructive interference between waves. If two states of an atom are coupled through several possible alternative transition processes, an interference between the amplitudes of these processes may lead to an enhancement (constructive interference) or a complete cancellation (destructive interference) in the total transition probability. These effects arise because in quantum mechanics it is probability amplitudes (which may be positive or negative in sign), rather than probabilities, that must be summed to obtain the total transition probability of a process. An example of this in atomic systems is Fano interference, seen for radiative transitions to autoionizing states, leading to asymmetric spectral profiles. This type of interference is at the heart of much of the recent work concerned with the laser-induced control of the optical properties of gaseous media, a subject pioneered by Steve Harris at Stan-

ford University.

How this applies to the two new experiments is shown in Figs 1 and 2. We are concerned with the optical properties, refraction and absorption, of the medium as seen by a weak 'probe' laser field in the absence and in the presence of the primary laser's strong field. The probe frequency lies near the frequency of the transition between atomic states 1 and 2 of rubidium (at 780 nm), and the primary laser's frequency is resonant with the state 2 to state 3 transition (at 776 nm). The importance of the strong 'coupling' laser field is that it induces, in effect, an infinite number of pathways for the transition from state 1 to state 2. This is an example of coherent excitation of an atom, in which although no significant population is ex-

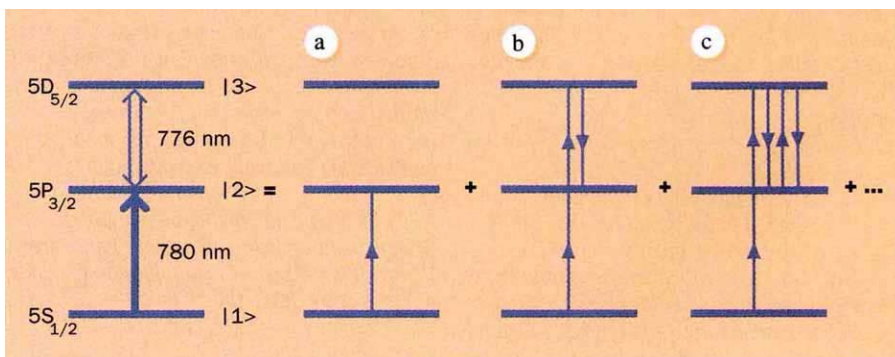


FIG. 1 The origin of laser-induced transparency in rubidium gas is most fundamentally viewed in terms of interference. In the presence of a weak probe laser (of wavelength 780 nm) and a strong coupling laser (at 776 nm) a number of alternative routes to the eventual excitation of state $|2\rangle$ are possible. Process a is simple single photon excitation to state $|2\rangle$; b represents a process involving also absorption and stimulated emission in the coupling field; c involves two absorptions and stimulated emissions, and so on. The amplitudes for the processes a, b, c, ... must be summed to generate the total probability amplitude (which is then squared to compute the total excitation probability). The signs of the amplitudes, for instance, of processes a and b are in fact opposite, so there is destructive interference in the transition probability. Overall, such interference can drive the absorption of the probe beam down to zero, and (more subtly) increase the refractive index at the same wavelength.

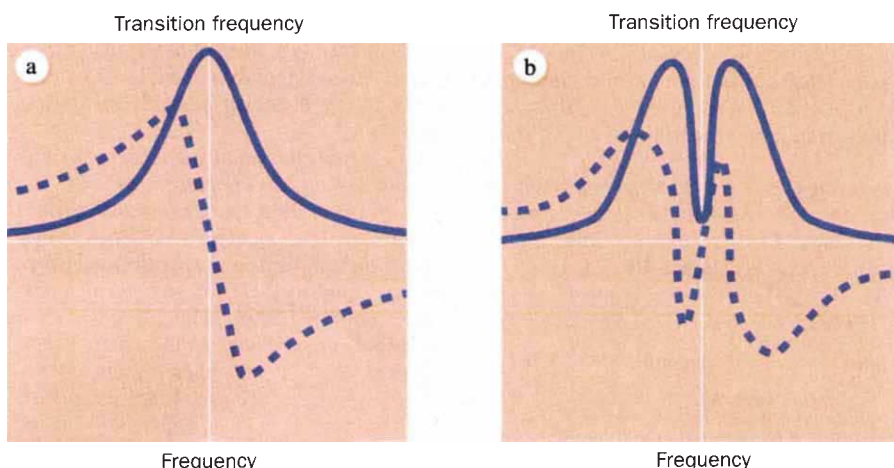


FIG. 2 *a*, Schematic diagram of the normal absorption (solid line) and refraction (broken line) near a resonance (like that at 780 nm in rubidium). Here the absorption shows a single, Doppler-broadened peak with high absorption loss near the line centre. The refraction shows a characteristic dispersion profile with a rapid variation (from positive to negative values) around the central frequency of the transition but this is exactly where absorption losses are high. *b*, The contrasting situation for the case with a strong coupling field at 776 nm. The absorption peak is now split with a region of vanishing absorption at the central frequency. The dispersion profile shows additional structure here, in particular a rapid variation in exactly the region of low absorption. This is the origin of both the increased refractivity and the induced lensing described in refs 1 and 2.

normally too small to achieve this, even if the laser beams are focused. This is where careful choice of the gaseous medium by the experimenter comes in. In the rubidium scheme, the near equality of the two laser wavelengths involved (780 and 776 nm) permits an experiment that is almost free from Doppler effects. The laser beams can be counter-propagated in the rubidium vapour so that the Doppler shift 'seen' by a particular atom is nearly equal and opposite for the two beams and thus effectively cancelled.

In the experiment of Xiao *et al.*¹, appropriately tuned laser diodes were used. They measured the refractive index changes near to resonance for the weak probe beam at 780 nm by means of a Mach-Zehnder interferometer. Their results confirm the predictions of theory, and further show that considerable changes to refractive index can be achieved using even relatively low-power diode lasers.

The work of Moseley *et al.*² goes somewhat further. They studied the spatial quality of the probe laser beam transmitted through the rubidium vapour, and observed convincing evidence of focusing and defocusing of the probe laser beam as its detuning was varied close to the resonance. Their observations were consistent with the lensing that would be expected given the spatial distribution of the coupling laser intensity and hence the spatial variation in the induced refractive index. The sign of the induced lens depends on the detuning of the probe laser, as this determines the sign of the induced refractive index gradient between the centre and the edge of the laser beam. The strength of the lens depends only on the strong laser

field intensity. Moseley and collaborators also observed laser-induced deflection of the probe beam, indicating the possibility of steering as well as focusing of one laser beam by the field of another.

Applications that await these enhanced refractivity systems might include ultrasensitive magnetometers⁶, high-sensitivity microscopy using a medium with laser-enhanced refractive index (rather than, for example, oil immersion) and compact particle accelerators⁷. But first there remain a number of practical problems to solve, not least the general problem of Doppler broadening.

Another important question, currently being addressed theoretically, is whether the optical properties of solid-state materials (for instance semiconductor quantum-well structures) can be similarly altered⁸. The proof-of-principle experiments suggest an exciting future for the laser control of the optical properties of a medium via coherent excitation. □

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DAEDALUS

Gore no more

EVEN a minor cut or abrasion leaks blood. Major surgical operations can lose so much blood that transfusions are often needed to replace it. Sometimes the patient's own blood is used — either withdrawn beforehand and stored, or recycled during the operation. Daedalus now plans to prevent the blood escaping in the first place.

Blood leaks out of a wound under its own blood pressure. To stop it, says Daedalus, simply pressurize the air over the wound to a slightly higher value. You might think that this would drive air into the tissues, with disastrous results. But Daedalus is unworried. Most blood leakage, he points out, comes from the capillaries. Their blood pressure is only about 0.05 atmospheres, but they are so narrow that capillary action would exclude air up to a pressure of 0.15 atmospheres. The bigger blood vessels lack this protection, but their internal pressure is about 0.15 atmospheres anyway, and surgeons routinely clamp the bigger blood vessels. So, says Daedalus, surgery conducted under a mere 0.1 atmospheres of overpressure should be effectively bloodless.

At first he envisaged a sort of pressurized tent sealed round the incision, with inset rubber gloves for the surgeon's hands. But this would need a tricky air-lock to enable clamps and scalpels to be passed in, and excised tissue out. It would be better to put the whole patient in a sort of 'iron lung' container, seal the region of the incision to a hole in its wall, and pump the container down to 0.9 atmospheres. The surgeon and his team could then work in the open, while the anaesthetist handled the circulation of air and anaesthetic gas to the patient in the container. All the problems of blood loss and top-up transfusion would vanish, and the surgeon would have dry, readily visible tissue to work on.

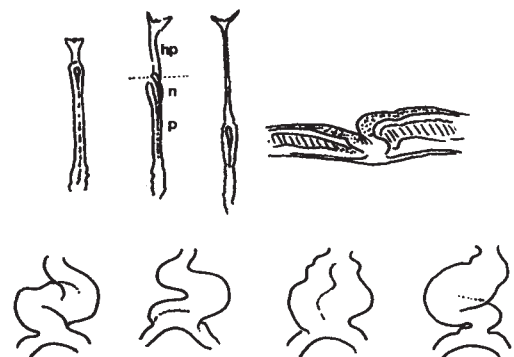
This scheme is so appealing that Daedalus wants to extend it to the more casual cuts and abrasions that never get inside a hospital. For these everyday nuisances, he is inventing a pressurized sticking-plaster. His first prototype had to be kept pumped up by a little lever, but he is now designing a much neater, self-pressurized plaster. It contains a volatile antiseptic such as ether, whose vapour pressure keeps the blood from leaking out until the scab over the wound has hardened sufficiently. Careless shavers, clumsy handymen and incautious glaziers will rush to order the new product. The small wounds of everyday life will soon be as bloodless and quickly healed as the more drastic incisions of surgery.

David Jones

Vertebrate embryo handedness

SIR — There has recently been renewed discussion of the various more or less consistent left–right asymmetries, that is the handedness, of the vertebrate body (see ref. 1). A strong but transient handedness is consistently visible in the bird embryo at stages well before formation of any of the organ systems. The chick Hensen's node, a structure where dorsal axial tissue for the future body is being fed into position in a head-to-tail sequence, assumes a 'handed' structure for just two to three hours. The tissue leaving the node at this time will form the mid- to hindbrain regions of the future head, where obscure asymmetries of structure in nervous system and pharynx roof are found, especially in the vertebrate relatives the protochordates². When three or four somites have segmented in the chick, apparent symmetry is regained, to last for many hours until appearance of the first permanent but localized handedness, that of the heart. The node handedness has been recorded by others, notably Lepori³ for the duck blastoderm, but information about its stability to developmental disturbance or relationship with subsequent handed phenomena is lacking.

During the asymmetry, the front end of the primitive streak is diverted towards the left as viewed from above, while the anteriorly emerging notochordal rod — the vertebrate midline structure — has its root at the right, before bending to adopt a course that extends the overall line of the streak or even swings rightwards.



Sketches show the following: upper row from left to right first shows the appearance of chick gastrulation from dorsal aspect, at early stage 5, stage 6 and early stage 8 (ref. 5). The node (n) regresses to shorten the primitive streak (p) and extend the head process (hp). Only when the fan-shaped prechordal tissue is already in place, but just the anteriormost notochord region has left the node, is pronounced asymmetry seen. Sketch at extreme right shows a typical transverse section tracing through the asymmetrical node, orientated as if an observer were facing anteriorly. Asymmetries are obvious in node tissue, in flanking mesoderm (the middle layer) and in overlying midbrain-level neural plate. The lower row shows in dorsal view, from left to right, two versions of the normally handed heart tube at the 12-somite stage, one with unclear handedness, and one with reverse handedness.

There are also marked differences in thickness and form of the neural plate and the sheet of emerging mesoderm on either side at the node (see figure). I have observed no instance of reversal or absence of this appearance in almost 150 blastoderms dissected from the egg at these stages, and it is independent of mechanical tensions that normally stretch the blastoderm, since it is maintained for hours in free-floating, living specimens.

The occurrence of individual bodies with various unusual combinations of left–right asymmetry in the developed organ systems (for example, heart, viscera, human brain function) may indicate that variously reliable developmental mechanisms derive each visible handedness from a hidden primary one, which underlies the consistent asymmetry normally found¹. Ring culture of chick blastoderms stretched on the inverted vitelline membrane⁴, from stages before and during node asymmetry, has consistently resulted in around 7% reversal of heart handedness. I have therefore cultured blastoderms from primitive-streak stages before the definitive node has developed, scored individuals for node handedness when this stage is reached, and ultimately examined the same individuals for heart handedness.

Normal node handedness was exhibited by every one of 75 such blastoderms at head-process stages, even though these were reached in a delayed way 7–12 hours after onset of culture, and the whole node region was often miniaturized and contained abnormally few cells. However, when examined at the 13-somite stage a further 20 hours later, 6 embryos had clear *situs inversus* of the heart, whereas a further 7 had hearts of abnormal symmetry or unclear handedness (see figure). Early gastrular handedness thus seems a more stably developing aspect of the bird embryo's structure than that of at least one later organ, the heart. Heart situs is in fact most liable to perturbation when embryos are placed in culture late, at times between the re-symmetrization of the node and actual heart development. It can thus be determined subsequent to, as well as potentially independently of, node handedness.

Viewed as if it were a primitive adult organism, the chick embryo rudiment at its most strongly handed stage would not be considered as primarily bilaterally symmetrical. Certain fossil evidence suggests that the chordate phylum evolved from a truly bilaterally symmetrical organism that 'fell over' on its original right-hand side, to re-acquire a

new more superficial left–right symmetry by progressive modification of the former dorsal (new right) edge of the body. One might indeed fantasize that in this very robust present-day handed structure, the bird gastrula displays its origins via dextrothetism¹, the application of a former right side to the substrate. Transient gastrular handedness can be easily observed in the translucent bird blastoderm, but should be actively searched for in other vertebrate types.

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Pi in the sky

SIR — The notion that at root "all is number" is a belief that can be traced back at least as far as the Pythagoreans, in around 500 BC¹. Although our current scientific conception of the Universe is indeed mathematical, it is hardly of the unalloyed simplicity conceived by the Ancient Greeks. But the mere appearance of the night sky can be used to deduce a remarkably accurate value for the transcendental number π .

This surprising result follows from an application of theorems in analytical number theory. For any integer p , the number of smaller integers with which p shares no factors is $\phi(p)$, where ϕ is the Euler totient function². Now, let (p, q) be a pair of integers chosen at random such that $q > 0$, $1 \leq p \leq q \leq n$, where n is large. There are of the order of $n^2/2$ pairs of such integers, and the number of them whose members are relatively prime is $\Phi(n) = \phi(1) + \phi(2) + \dots + \phi(n)$. The probability that two random integers are relatively prime is thus $2\Phi(n)/n^2$, where for large n it can be shown that²

$$\Phi(n) \approx n^2/2\zeta(2) \quad (1)$$

with $\zeta(2) = \pi^2/6$, the value of the Riemann zeta-function for reciprocal squares. Thus the probability that two integers chosen at random are relatively prime is

$$P(p, q) = 6/\pi^2 \quad (2)$$

This result enables us to calculate a value of π from a large random sample of integers: we form pairs of such integers, and determine what proportion are relatively prime.

The scattering of the brightest stars over the night sky suggests they may allow a 'celestial' estimate of π . Let (α_i, δ_i) be the right ascension and declination of the i th star in the night sky. Its angular distance from the j th star is then Δ_{ij} , where³

$$\cos\Delta_{ij} = \sin\delta_i\sin\delta_j + \cos\delta_i\cos\delta_j\cos(\alpha_i - \alpha_j) \quad (3)$$

These angular distances may be turned into a source of random integers r_i distributed over the range $(0, 10^k)$ via the transformation

$$r_i = \text{integer}[10^k(1 + \cos(\Delta_{ij}))/2] \quad (4)$$

The relative primality of two such integers (r_i, r_j) can then be determined by the standard euclidean algorithm for finding greatest common divisors², and a value of π derived from the proportion of such pairs having greatest common divisors of unity.

Using the astrometric data for the 100 brightest stars in the sky given in ref. 4, we computed the corresponding 4,950 values of $\cos(\Delta_{ij})$, and from them derived 10^6 pairs of random integers (r_i, r_j) in the range $(0, 10^6)$. We found that

$$P(r_i, r_j) = 0.613333,$$

so that, by equation (2),

$$\pi_{\text{Stars}} = 3.12772$$

which is within 5 parts per 1,000 of the standard value. Latter-day Pythagoreans may take encouragement from learning that a 99.6 % accurate value for π can be found among the stars above their heads.

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Superconductivity of boro-nitrides

STR — The discovery of superconductivity with critical temperatures reaching $T_c = 23$ K in a new class of compounds has reinvigorated searches for high-temperature intermetallic superconductors^{1,2}. $\text{LuNi}_2\text{B}_2\text{C}$ is prototypical^{3,4}, consisting of flat nickel layers closely coordinated by boron atoms. These Ni_2B_2 sheets are separated by LuC layers, yielding a layered structure. This structure, combined with the presence of potentially magnetic Ni, is reminiscent of the very high- T_c copper-oxide superconductors and has added to the excitement. However, theoretical studies^{5,6} have shown that, from the point of view of their electronic structure near the Fermi energy, the materials are quite three-dimensional and more closely related to previous families of intermetallic superconductors.

The subsequent discovery⁷ of superconductivity at 12–13 K in the structurally related material $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ is remarkable. This compound may be derived from $\text{LuNi}_2\text{B}_2\text{C}$ by replacing the LuC layers by three LaN rock-salt layers while keeping the Ni_2B_2 sheets intact. The result is much

more widely separated Ni_2B_2 sheets, with nominally insulating LaN trilayers in-between. The natural expectation is of considerably more two-dimensional behaviour, possibly pointing the way to much higher T_c by analogy with the cuprates. The crystal and electronic structures and the phonons are crucial to understanding the superconductivity.

The reported crystal structure determined primarily by electron diffraction⁸ is, however, anomalous. In particular, the B height above the Ni planes is much smaller than in the boro-carbides: 0.60 compared with 1.19 Å in $\text{LuNi}_2\text{B}_2\text{C}$. We have used first-principles methods, as in our earlier studies of $\text{LuNi}_2\text{B}_2\text{C}$, to analyse the properties of $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$. The three internal coordinates not fixed by symmetry have been calculated along with the corresponding Raman optical phonon frequencies. The lattice parameters were fixed at their accurately determined X-ray values⁷. The atomic positions (notation of Zandbergen *et al.*⁸) are 0.131 for La (2), 0.128 for N (2) and 0.199 for B. This corresponds to a B height of 1.05 Å, much more like $\text{LuNi}_2\text{B}_2\text{C}$, and a more reasonable B–N (2) distance of 1.46 Å (compared with 1.81 Å). The calculated energy with the positions of Zandbergen *et al.*⁸ is 2.2 eV per formula unit higher — well outside plausible errors caused by the local-density approximation. We find that the structure of Zandbergen *et al.* is incorrect, perhaps because of reliance on electron diffraction measurements.

The fully symmetric A_{1g} Raman vibrations in the harmonic approximation are an almost pure La mode at 106 cm^{-1} , and two strongly mixed B–N modes at 323 cm^{-1} and 896 cm^{-1} . The upper mode has adjacent B and N atoms moving against each other. The upper, but not the middle, mode is strongly coupled to electronic states near the Fermi energy. This may be important for superconductivity, although the different couplings of these two modes, both of which involve modulation of the NiB_4 tetrahedral bond angles, suggests a more complex mechanism than that proposed by Mattheiss and co-workers⁹. The electronic structure near the Fermi energy is somewhat more anisotropic than that of $\text{LuNi}_2\text{B}_2\text{C}$, but cannot be described as even quasi-two-dimensional. The ratio of the Fermi velocities in the x and z directions is only 2:1.

The similarity to the boro-carbides is the result of unanticipated incomplete ionization of La and therefore metallic LaN layers in this structure.

Thus, $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ is well described as the first member of a subclass of the $\text{LuNi}_2\text{B}_2\text{C}$ class of intermetallic superconductors. These materials appear much more similar in their electronic properties to previous families of intermetallic superconductors than to the high- T_c copper-oxide materials.

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ZANDBERGEN AND CAVA REPLY — Our original reported structure for $\text{La}_3\text{Ni}_2\text{B}_2\text{N}_3$ (ref. 8) did contain some inaccuracies. Since that paper was submitted, three types of structure determination have been used to obtain an improved refinement of the crystal structure of that phase. They are: total-profile neutron powder diffraction¹⁰; through-focus exit-wave reconstruction using a series of high-resolution electron microscope images¹¹; and refinement of the electron-diffraction data in the original paper that now takes into account the effects of dynamical diffraction (J. Jansen *et al.*, manuscript in preparation).

The atomic positions obtained by these three methods are approximately the same, and broadly agree with those calculated by Singh and Pickett. But comparison with the structure reported in ref. 8 shows significant differences in the positions of the B atoms ($z=0.195$, as compared with $z=0.221$ in ref. 8) and N atoms ($z=0.125$, as against $z=0.133$ in ref. 8). As refinement of the original electron-diffraction data including dynamical diffraction gives results similar to those of neutron powder diffraction measurements, the inaccuracies in the positions of B and N in ref. 8 must be due to the neglect of the dynamical diffraction effects. (This was noted as a possible complication in that paper.) At the time our paper⁸ was submitted, only the kinematic refinement could be performed.

On the other hand, the positions of the Ni and La atoms reported in ref. 8 are the same (within the stated errors) as those obtained by the three new methods. We anticipate that within a few years, quantitative electron diffraction (taking into account dynamical diffraction) will provide a quick way to determine crystal structures from small crystallites which does not require the growth of bulk single crystals or high-purity materials.

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All plant life is there

Robert Shields

Arabidopsis. Edited by Elliot M. Meyerowitz and Chris R. Somerville. Cold Spring Harbor Laboratory Press: 1995. Pp. 1,300. \$175.

OPEN any of the glossier plant molecular biology journals and many of the articles feature *Arabidopsis*. Look in any journal of plant physiology, biochemistry or pathology and this tiny plant hardly rates a mention. Although *Arabidopsis* has become the virility symbol for plant molecular biologists who argue it is not size but what you do with it that counts, the wider plant community remains less than convinced.

The virtues of *Arabidopsis* for genetic studies — its short generation time, small size, prolific seed production and ease of chemical mutagenesis — are well rehearsed in this volume. The molecular biology really took off with the demonstration that genes could be simultaneously mutagenized and tagged by *Agrobacterium*-delivered T-DNA and with the realization that high-density genetic maps based on restriction-fragment-length-polymorphisms and the small genome size made positional cloning a possibility. The genome size is generally reckoned to be about a hundred megabases, the same size as that of the nematode *Caenorhabditis elegans*, seven times larger than that of baker's yeast but about a thirtieth of that of maize or man. And like yeast, *C. elegans* and man, *Arabidopsis* has spawned its own genome projects. At least two groups are randomly sequencing complementary DNAs to build libraries of expressed sequence tags and others are limbering up to sequence the entire genome.

Yet results from the yeast and the *C. elegans* genome projects already show us what the results of an *Arabidopsis* genome project are likely to be: large numbers of genes only a third of which have homology to genes in the database (and so whose functions can be guessed) and most whose function will be obscure. At least in yeast a specific gene of unknown function can be selectively mutated by homologous recombination to see what effect its deletion has (none in most cases); targeted gene knock-out is some way off in plants. We already know from T-DNA and transposon-tagging experiments that most non-targeted insertions into the *Arabidopsis* genome (and many of these will be in genes) fail to produce a phenotype (or at least one that is evident to a molecular biologist). To paraphrase an apposite quotation from Sydney Brenner (admittedly made in 1982 — I wonder if he believes it still?), molecular biologists study known genes

and their known products, biochemists study the known products of unknown genes, geneticists study known genes and their unknown products, only lunatics study the unknown products of unknown genes. Could it be that the impetus for these genome projects comes more from the desire of granting agencies to have projects with 'timescales', 'milestones' and 'deliverables', to use the currently fashionable management Newspeak, rather than projects that approach interesting scientific questions? For there are plenty of those, as this volume amply demonstrates.

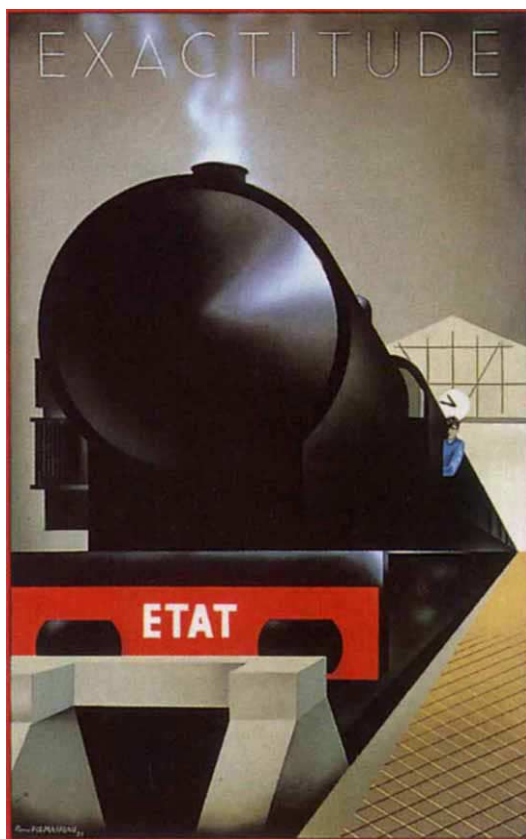
Thanks to efforts of molecular biologists working with mutants of *Arabidopsis*, we have discovered in the past few years a lot more about the mechanism of action of the plant hormones, circadian rhythms, response to light and many other topics than had been learned by plant physiologists in the previous decades. And the pace is accelerating, so much so that even though the papers in this volume date from 1994, some have

been overtaken by events.

Apart from what can be learned about what makes a plant tick, the genetic resolution offered by *Arabidopsis* can give insights into questions of interest to plant breeders. What for instance is the genetic basis of heterosis (where a hybrid plant is superior to the parental lines)? Is it due to single genes with true heterozygotic advantage (as hybrid-seed merchants would have one believe) or closely linked genes having simple dominant alleles in a *trans* configuration? What of quantitatively inherited traits (that is traits governed by several genes with different effects and influenced by the environment) and which are so important for plant improvement? Because one can have absolute control over the environment of *Arabidopsis* (just alter the conditions in the incubator), these plants offer the possibility of resolving quantitative traits into their individual genes.

This book, running as it does through plant systematics, genetics, developmental biology, hormones, the chloroplast, pathology, the cell wall, metabolism and many other topics, should be dipped into by plant physiologists, biochemists and breeders and should convert doubters to the virtues of the weed. □

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Exactitude by Pierre Fix-Masseau. The drawing is reproduced on the cover of *The Value of Precision* edited by M. Norton Wise, which looks at the role of precision in the evolution of science and its importance in Western culture. Written by distinguished historians of science, the essays support the view that centralized states — with their increasingly widespread bureaucracies for managing trade, taxation and armies — and large-scale commercial enterprises — with their requirements for standardization and mass production — have been the key promoters of numerical exactitude. Topics range from the measurement of the population of France to the electrical resistance of copper to the solvency of insurance companies. Princeton University Press, \$49.50, £35.

A question of Y?

Ursula Mittwoch

Sex Determination, Differentiation and Intersexuality in Placental Mammals. By R. H. F. Hunter. *Cambridge University Press*: 1995. Pp. 310. £50, \$79.

In *The Genetical Theory of Natural Selection*, published in 1930, R. A. Fisher suggested that the difference between the sexes might depend on a single Mendelian factor that switches on one or other possible alternative. Admittedly, males and females in both humans and the fruitfly *Drosophila melanogaster* differ not just in one gene but in a whole chromosome, but it seemed that the sex of platyfish might be determined by a single gene. Because sex in *Drosophila* is primarily determined by the number of X chromosomes, the chance of finding such a gene in this species was rather remote. But the discovery that in humans and other mammals the Y chromosome is closely associated with males once again opened the possibility of finding the sex-determining gene, which was named *TDF* for testis-determining factor in humans, and *Tdy* in the mouse.

The name reflects the idea publicized by Alfred Jost that male development requires hormones produced by the fetal testis, whereas female development happens automatically in the absence of these hormones. Intense research during the past 20 years has resulted in proposals of several candidate genes for *TDF*. Following the announcement that the insertion of a small piece of DNA containing the murine homologous gene into genetically female XX mouse embryos is successful in inducing male development in some of them, the *SRY* gene is today widely accepted as the testis-determining gene.

This book provides a clear and factual account of the experimental evidence that has led to the present position, against a background of normal and abnormal sexual development. It contains ten self-contained chapters, from "Historical landmarks in studies of reproduction and sex determination" to "Concluding thoughts and a current perspective", and although fully referenced, the book is very readable. My one criticism is the absence of scale bars from some of the illustrations.

Ronald Hunter is a reproductive biologist specializing in farm animals, his species of choice being the pig. Pigs produce many eggs at ovulation and often show abnormalities of sexual development, such as the presence of ovotestes or of an ovary on one side and a testis on the other. These animals, which are a source of financial loss, are classified as 'inter-sexes', whereas the same abnormality in humans would be referred to as 'true hermaphroditism'. The difference in terminology

reflects the fact that the biology of domestic animals is seen in the context of that of simpler organisms, in which 'hermaphroditism' implies the ability of individuals to produce both functional eggs and sperm. By contrast, fertility is not a requirement for gender assignment in humans, and the clinical term 'true hermaphroditism' is applied to individuals with testicular and ovarian tissue, neither of which is reproductively competent. In this respect, mouse terminology follows the human practice.

Most intersex pigs are genetic females, with two X chromosomes and apparently no Y-chromosomal material; nevertheless,

owing to the formation of vascular anastomoses between the two fetuses, and he postulated that humoral factors passing from the male to the female twin cause the latter's intersexual development. The reason the female becomes masculinized, whereas the male does not become feminized, Lillie suggested, is that sexual differentiation occurs earlier in the male than in the female.

Freemartins are rarely found in other species such as sheep and goats, and the condition seems to be absent in primates. In human pregnancies with opposite-sexed twins, the female twin develops normally.

Nevertheless, humans have their fair share of sexual abnormalities, and some of these have provided the basis of our knowledge of sex determination. The discovery that men with Klinefelter's syndrome have XXY sex chromosomes, whereas women with Turner's syndrome have a single X and no Y, has led to the appreciation that the Y chromosome is involved in the determination of male sex, and various deletions of the Y chromosome have provided information on the location of genes for sex determination (*SRY*) and for fertility. But there are a few males who lack Y chromosomal genes and a few females with an intact Y chromosomes. To explain these exceptional individuals, the effects of genes on other chromosomes are invoked.

In mice, several genetic causes are known that give rise to XY females, one of them resulting from crosses between wild mice of the *domesticus* substrain with the inbred laboratory strain C57BL/6J. When the *domesticus* Y chromosome finds itself in the company of the chromosomes of this strain, it is incapable of inducing the development of normal testes, apparently because its testis-determining gene (*Sry*) acts too late and is forestalled by ovarian development. But what causes the delay? Recent evidence suggests that this gene is already transcribed in the embryos immediately after fertilization, when male embryos are already further advanced than female ones. Is there a connection between these findings, and what is their relevance to the process of sex determination? Also, in this day and age, should not more active consideration be given to the positive steps involved in the formation of the ovary, rather than regarding it as the result of a default pathway?

Hunter sets out the relevant facts and hypotheses in a perfectly detached manner, allowing readers to make up their own minds. The book is eminently suitable for the advanced students for whom it is intended, and is a must for their teachers, as well as for anyone involved in the field of mammalian sex differentiation. □

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IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Mixed blessing — a hermaphrodite, as depicted in an erotic mural from Pompeii.

some exhibit the aggressive behaviour of boars, indicating that although the testicular tissue lacks germ cells, testosterone secretion takes place and affects behaviour. To explain the masculinization, a 'downstream' mutation has been invoked, but it is less easy to account for the common finding of unilaterality, with an ovary on the left side and an ovotestis on the right side.

A similar intersexual condition is found in 'polled', or hornless, goats. The P gene causes hornlessness when in single dose, whereas in double dose it also causes testicular development in genetic females.

Probably the most famous intersex is the bovine freemartin, which many decades ago helped to lay the foundation of the hormonal theory of sex differentiation. A freemartin is a sterile heifer born as co-twin to a male calf. During gestation, the ovaries of the female fetus lose their germ cells and sometimes develop into testicular structures. About 80 years ago, Frank Lillie noticed that freemartins are formed only if the twins share a common circulation

Mary Evans

Tetrapod tracking

David Weishampel

In the Shadow of the Dinosaurs. Edited by Nicholas C. Fraser and Hans-Dieter Sues. Cambridge University Press: 1995. Pp. 435. £50, \$89.95.

DURING the Mesozoic era, dinosaurs cast a long shadow — much as they do in today's media — but the terrestrial world throughout that time was not overly encumbered by these behemoths. We now know that there was a vibrant community of small Mesozoic tetrapods and that in fact it was in the context of this amalgam of diminutive competitors and ancestors that dinosaurs had their origin roughly 230 million years ago during the Late Triassic period. But terrestrial life burgeoned in other ways during the Late Triassic and into the Early Jurassic. Many extant groups of tetrapods — birds, crocodiles, squamates, turtles — trace their common ancestry to this time interval. Because it was also a time marked by the appearance of the first flying vertebrates (pterosaurs) as well as important mammal progenitors, the transition from the Triassic to the Jurassic is rightly considered to be a pivotal time of biological experimentation in the terrestrial world.

In May 1991, an international group of researchers met at Front Royal, Virginia, to consider this transformation of the terrestrial biota; the results are presented in this important book containing 25 chapters by 35 specialists on the fossil record, palaeoecology and evolutionary dynamics of the tetrapods from the Late Triassic and Early Jurassic.

The book rightly begins by emphasizing the fossil record and phylogenetic context of the radiation of tetrapods during the Triassic–Jurassic transition. These studies — on Amphibia (Milner), Lepidosauromorpha (Rieppel), Sphenodontia (by Wu and by Carroll and Wild), Crocodyliformes (Clark) and mammalian progenitors (Luo) — are fundamental in establishing the pattern of descent among these new terrestrial inhabitants and in discerning the features of the basal members of respective clades. Several of these studies are more than descriptive anatomy, taxonomy and presentations of phylogenies; they represent explicit phylogenetic analyses (Wu, Clark, Luo), a healthy trend in any kind of phylogenetic work.

Although focusing first on phylogeny, the book is dominated by chapters on faunal assemblages. Ranging widely from the Middle Triassic of Britain (Benton *et al.*) to the Middle Jurassic of Mexico (Clark *et al.*), these studies emphasize assemblage diversity (often with faunal lists), local and regional stratigraphy and often

palaeoecology and palaeobiogeography. All of these aspects of Triassic–Jurassic faunistics rely heavily on the considerable research on the exceptional fossil record of the eastern seaboard of North America. Most of it originally done (and presented) by contributors to this volume, this work is rightly seen as the driving force behind much of the new interest in the Triassic–Jurassic transition.

The final section of the book tackles a wide variety of issues, including stratigraphic and biogeographic aspects of faunal change, Late Triassic palaeoclimatology, extinction and evolutionary rates. Particularly interesting is the continuing debate about one or more mass-extinction events at the end of the Triassic. Revolving around how many, timing and cause(s), Late Triassic extinction patterns are tackled from the perspective of taxonomic sampling, biostratigraphy and sedimentology. Yet even with these approaches, there seems little resolution of either the tempo or mode of biotic dynamics at the end of the Triassic (compare Simms *et al.*, Benton, Fraser and Sues, and Padian).

In setting out current knowledge of Triassic–Jurassic transition among terrestrial tetrapods, the book reviews and resolves a number of old questions (advances in information on Early and Middle Jurassic faunas from around the

world, biostratigraphic and geochronologic refinements across the Triassic–Jurassic transition and others). Yet, like any work of significance, it also explores unresolved topics and even raises several new questions. How many extinction events were there by the end of the Triassic? What was their severity and selectivity? How did the integration of phylogeny, faunistics and stratigraphy affect these patterns? And is there any possibility of implicating the driving force behind the dynamism between evolution and extinction?

However these questions are answered (and they are bound to generate important research in the future), the Triassic–Jurassic transition has had a lasting impact on land-dwelling organisms. Although it is common to look to the extinction that swept away non-avian dinosaurs 65 million years ago for the rise of modern terrestrial ecosystems, perhaps it is more appropriate to look back roughly 140 million years earlier. As the editors and many of the authors of this book argue, nearly all modern tetrapod clades owe their existence to events — biological and otherwise — of those ancient times. □

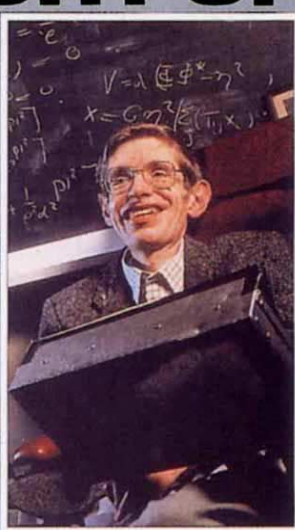
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A BRIEF HISTORY OF TIME

FROM THE BIG BANG TO BLACK HOLES

STEPHEN W. HAWKING

INTRODUCTION BY CARL SAGAN



ABOUT time — the UK paperback edition of Stephen Hawking's *A Brief History of Time*, originally published in 1988, has finally seen the light of day (Bantam, £6.99). Reprinted nearly 50 times, the UK hardback edition has sold more than 600,000 copies (worldwide sales exceed 8 million). An interactive CD-ROM that integrates the complete text of the book with graphics, movies and animations is also available (W. H. Freeman, \$59.95, £49.95).

Past master

Paul G. Bahn

Time Detectives: How Archeologists Use Technology to Recapture the Past.

By Brian Fagan. *Simon and Schuster*. 1995. Pp. 270. \$24, £17.99.

As one has come to expect from Brian Fagan, a leading popularizer of archaeology, this new collection of essays is lively and highly readable. It comprises a wide variety of topics, many of which he has recently covered in the magazine *Archaeology* in feature articles or in his regular column. But he gives us no indication why these particular subjects have been chosen for inclusion in the book, and one can only

Fagan has been ill-served by his publishers in one respect: the text is illustrated with only a few line drawings, while at the centre of the volume is a collection of 20 small, mostly uninspiring, black-and-white photographs, making it look like the kind of book published in the 1950s or 1960s. The text, by contrast, is vivid and often gripping, with only an occasional oddity, such as the description of Pueblo Bonito as "a Sistine Chapel of Native American architecture". Fagan's one weak point seems to be names: the sites of Dos Pilas and Olsen-Chubbuck are misspelled throughout; Yuri Knorosov is repeatedly called Knosorov; and fleece-expert Michael Ryder becomes Martin. But these are minor quibbles in what is otherwise an excellent book.

I was left with one final, personal source

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Out for a drive? American bison 1811.

assume that the limited geographical spread in this potpourri represents those parts of the globe with which he is most familiar — the New World, the United Kingdom, Egypt and the Near East. He presents 13 studies in these regions, ranging from prehistoric hunter-gatherers to the gardens of eighteenth-century Annapolis in Maryland, and from American bison-kills to Hadrian's Wall across the north of England. Some of the material is easy to convey to the public, such as the exciting and spectacular finds from the Bronze Age shipwreck of Uluburun in the eastern Mediterranean or the written documents from the Roman fort of Vindolanda near Hadrian's Wall, but other topics are unprepossessing, and even Fagan, despite his talent for making difficult subjects palatable, is sometimes hard pressed to maintain the reader's interest with evidence for environmental change. As the subtitle indicates, the focus is on how archaeologists use new technology to obtain information, but this is not a consistent theme, as some essays, such as the bison-kill study, involve no such technology. The book's excellent title, however, is both apt and evocative.

of puzzlement: how Fagan, a scholar with a healthy degree of scepticism and who can be hypercritical of some evidence (notably in claims for early occupation of the New World), can swallow unquestioningly the conclusions of Tim White and others that the Anasazi of the American Southwest were cannibals. He seems to believe that there are scientific criteria for identifying anthropophagy in the archaeological record (I am unaware of any, other than finding human remains in human stomachs or faeces), that "White has documented cannibalism at Mancos" and that "without question, these Anasazi were eating human flesh". Needless to say, White has proved nothing of the kind, and other possible interpretations of the Anasazi bones have been swept aside or ignored (see P. Bullock, *Man* 29 (1), 190–191; 1994). This lapse of judgement, however, does not detract greatly from the quality and perspicacity of a book that can be warmly recommended to anyone seeking some idea of how modern archaeology works in a variety of settings. □

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Spring Books

Next week's issue contains *Nature's* Spring Books supplement. Reviewers include David Jones on nanotechnology, W. C. McGrew on the orangutans of Borneo, John L. Casti on reductionism, Stuart Sutherland on the history of multiple-personality disorder, George J. Annas on human experimentation in the United States, June Goodfield on Marie Curie, Roy Porter on landscape and memory, Paul R. Gross on science and anti-science, and Frank Gonzalez-Crussi on human physical disability and deformity in ancient Greece and Rome.

New Journals

This year, *Nature's* annual new journals review supplement will appear in the issue of 21 September. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals that first appeared during or after June 1993 and issued at least four separate numbers by the end of April 1995 will be considered.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.
- Deadline for submission is the end of May.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates. For further information please contact Peter Tallack, *Nature*, Macmillan Magazines Ltd, Porters South, Crinan Street, London N1 9SQ, UK. Tel: +44 (0)171 833 4000.

New in paperback

Conversations with Neil's Brain: The Neural Nature of Thought and Language

by William H. Calvin and George A. Ojemann. Addison-Wesley, \$12. A series of popular stories about the combined efforts of a surgeon and a neuroscientist to remove a portion of the temporal lobe of an epileptic patient's brain.

The Gnat Is Older Than Man: Global Environment and Human Agenda

by Christopher D. Stone. Princeton University Press, \$15.95, £12.95. An overview of the possible risks surrounding various environmental problems and what can be done about them.

The History of the Countryside by Oliver Rackham. Weidenfeld and Nicolson, £12.99. A classic history of Britain's landscape, flora and fauna.

Mary Evans

New evidence for extraordinarily rapid change of the geomagnetic field during a reversal

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Palaeomagnetic results from lava flows recording a geomagnetic polarity reversal at Steens Mountain, Oregon suggest the occurrence of brief episodes of astonishingly rapid field change of six degrees per day. The evidence is large, systematic variations in the direction of remanent magnetization as a function of the temperature of thermal demagnetization and of vertical position within a single flow, which are most simply explained by the hypothesis that the field was changing direction as the flow cooled.

THE Steens Mountain reversal record, dated radiometrically at 16.2 Myr before present¹, has long held the reputation as the best recording of a polarity transition by lava flows². A detailed study³⁻⁵ found 56 distinguishable flows with stable direction of magnetization intermediate between normal and reversed. Figure 1 shows this record, which can be divided into two parts. In the first, the field moves from stable reversed polarity through transitional directions to temporary normal polarity. In the second, the field retreats from the short-lived episode of normal directions back to intermediate directions that are similar to those attained before, and then successfully completes the transition to normal polarity directions via a different route.

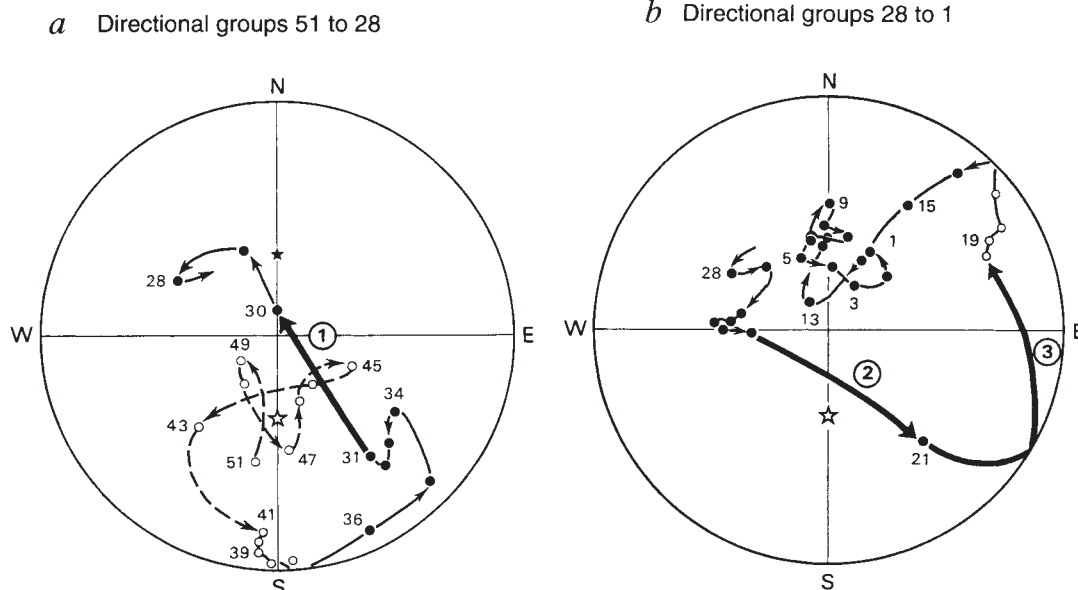
An interesting aspect of the record is the three large gaps, two of which occur in the second part (Fig. 1). Because we did not observe obvious signs at the outcrop of a lapse in eruption rate at any of the gaps, and because flows within two of the gaps yielded anomalously scattered directions for no apparent reason, we suspected originally that the field may have changed unusually rapidly during the time of the gaps³⁻⁵. Re-study of the flow in the first gap yielded evidence strengthening our suspicion, suggesting that the field may have changed extraordinarily rapidly (3° or 300 nT per day)⁶. However, we could not completely

rule out unremoved viscous magnetization as a possible alternative explanation. In this Article we focus on the second gap, recorded in a parallel section 1.5 km to the north, where the field jumped about 80° from westerly to southeasterly declinations (Fig. 1b). As we shall show later, viscous contamination is not a tenable explanation for the surprising results we obtained, nor do several other alternative thermal and rock-magnetic hypotheses stand up to scrutiny.

The new section

To carry out this study we moved 250 m north along the cliff from the original section A, tracing two prominent flows A38 and A39 (directional group 21 of Fig. 1) that outcrop continuously, to a place where the underlying flows are particularly well exposed and could be sampled vertically (section D, Fig. 2). There, these flows are laterally continuous on a scale of 100 m, in contrast to some of those in the same stratigraphic position at the original location, which pinch out within a few metres of the sampled sections. Thus some of these flows are probably not precisely the same units as at the original section, although a cover of rubble between the two places prevented us from verifying this directly. Nonetheless, just as was the case at the previous

FIG. 1 The Steens Mountain directional record showing the three large jumps or gaps (encircled numbers). The projection is equal area, and each point is a directional group that represents one to nine consecutive flows with indistinguishable directions. Stars denote normal and reversed geocentric axial dipole directions. Filled (open) symbols are plotted on the lower (upper) hemisphere. *a*, First part of record: field direction moves from stable reversed to transitional to temporary normal. *b*, Second part of record: field direction moves from temporary normal to transitional to stable normal.



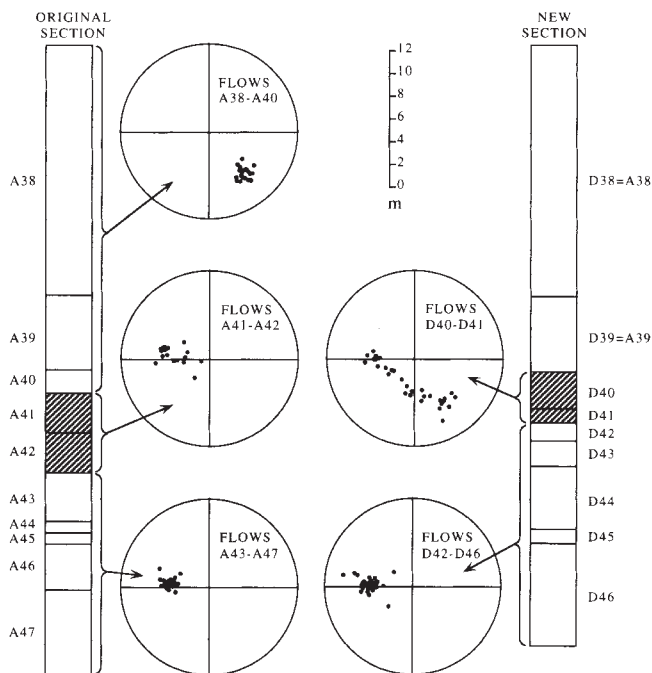


FIG. 2 Natural remanence directions after thermal demagnetization to 500 °C of samples from flows within the directional gap (shaded), which display anomalous scattering, and from pre-jump and post-jump flows. At the new section, where each flow was sampled along a vertical profile, flow D41 and D40 directions smear out all the way between the directions of the underlying and overlying flows. At the original section³, where horizontal coverage was stressed more than vertical sampling, flow A42 and A41 directions are nonetheless significantly smeared, with Fisher³⁸ precision parameter $k=29$ as compared to $k=196$ and $k=156$ for the pre- and post-jump flows below and above, respectively.

locality, the two flows D41 and D40 immediately underlying the prominent marker flows A39 and A38 fall within the same directional gap and display anomalously scattered directions of high-temperature remanence, which smear out between the tightly clustered directions of the flows immediately below and above (Fig. 2). Moreover, in contrast to samples from their margins and from other flows, many samples from the interior of flows D41 and D40 fail to reach a stable endpoint during thermal demagnetization; instead, their directions tend to rotate from southerly towards westerly declinations as the temperature progressed beyond 300 °C (Fig. 3a).

Even more remarkable, however, is the systematic variation of direction of high-temperature remanence of samples within flows D41 and D40 as a function of their vertical position (Fig. 3b). From bottom to top in the lower two-thirds of flow D41, the direction moves from near that of underlying flow D42 (pre-jump direction) about 60° towards that of the overlying flow A39 (post-jump direction), and then back to the flow D42 direction again. The swing in direction is corroborated in sections 2 m to the south and north, which overlap the bottom and top of the principal section. A similar but smaller swing occurs in the lower one-third of flow D40, but above that all samples exhibit stable endpoint directions that are close to those of the overlying flow.

Rapid-field-change hypothesis

A simple hypothesis to explain these results is that when flow D41 was cooling through its magnetic blocking temperatures, the geomagnetic field was moving from the pre-jump direction recorded by flow D42 towards that of the post-jump direction recorded by flow A39. This accounts very naturally for the change in remanence direction away from that of the overlying flow towards that of the underlying flow during progressive thermal demagnetization of individual samples (Fig. 3a). It also explains the observed variation in direction of high-temperature remanence with vertical position in the flow (Fig. 3b), because the more slowly cooled interior of flow D41 records directions that are further from that of the underlying flow.

The similar but less pronounced swing shown in the lower part of flow D40, which picks up approximately where that in flow D41 leaves off, suggests that it was erupted shortly after

D41 was emplaced, probably even before D41 had completely cooled. The samples higher in D40 appear to have been remagnetized by the overlying flow A39, because they all maintain directions close to those of A39 at every demagnetization step above 350 °C. They also exhibit titanomagnetite-granulation textures in polished thin section under reflecting light that are characteristic of hydrothermal alteration⁷. This texture is absent in the lower third of D40 and all of D41, suggesting that flow D40 may have protected D41 from being remagnetized by the hydrothermal action of the much thicker A39.

Experimental observations of cooling basaltic lava on the island of Hawaii demonstrate that simple conductive calculations can describe the evolution of temperature with time reasonably well, especially when an effective thermal diffusivity of $k = 6 \times 10^{-7} \text{ m}^2 \text{ s}^{-1}$ is used to take account of average vesicularity and other second-order factors⁸. The characteristic cooling time for a thin sheet of constant thickness and thermal diffusivity held at constant temperature at its boundaries is $t = a^2/k$, where a is half the thickness⁹. For flow D41, which is 1.3 m thick, this gives only 8 days, suggesting an astonishingly fast rate of change of field direction of the order of 10° per day. This rapid change apparently did not occur as a result of vanishingly low geomagnetic intensity during the directional jump. Palaeointensity experiments conducted previously⁵ yielded estimates of the transitional field intensity that decrease from 10 to 7 µT for the three pre-jump flows, remain at 7 µT for the two anomalous flows in the jump (samples from the quickly chilled flow margins), and increase from 4 to 13 µT for the three post-jump flows overlying the jump. Thus the ancient field strength during the cooling of anomalous flow D41 was probably about 7 µT, which would require a rate of change of total field around 1 µT per day to account for the directional rate.

Alternative hypotheses

This rate is so extraordinary, of the order of 1,000 times faster than that directly observed in secular variation of the geomagnetic field¹⁰, that we are obliged to consider carefully any alternative hypotheses that might account for our results. These are of two general classes: thermal (that is, simple baking or multiple cooling units) and rock magnetic (that is, anisotropic remanence acquisition, viscous contamination or chemical remagnetization).

Baking. Hypotheses involving simple baking by the overlying flow are beset by serious difficulties. Thermal demagnetization experiments show that only the upper two samples of flow D41 were significantly remagnetized by the overlying flows, exhibiting appreciable angular deviations of remanence between 300 and 500 °C from the pre-jump towards the post-jump direction. Beyond 500 °C thermal demagnetization, the directions of the six upper samples are very similar to the pre-jump direction, which implies that the upper 60 cm of the flow was not reheated above that temperature. Below 60 cm, the directions display the peak in angular deviation with depth (Fig. 3b) that gives rise to the rapid-field-change hypothesis. For this signature to have been caused instead by baking, the middle part of the flow would have to have been reheated to higher temperature than the upper 60 cm, which is of course unrealistic.

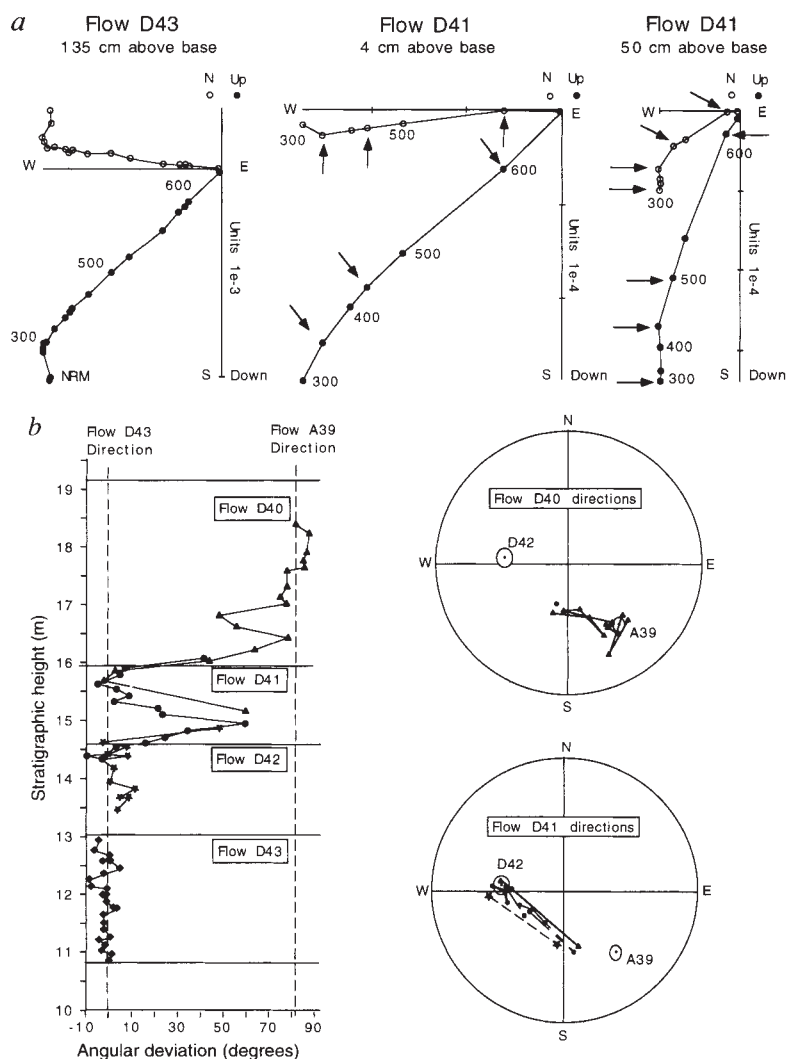
Multiple cooling units. More flexibility is afforded by the hypothesis that flow D41 was erupted as more than one cooling unit. To account for the directional signature of flow D41, any later-erupted unit would have to intrude the first horizontally, thereby delaying cooling of the interior relative to the margins. Examples of such behaviour are known from observations of modern eruptions, but unless intrusion occurs before most of the first unit has crystallized the two units do not weld at their boundaries and the compound nature of the flow is apparent (G. P. L. Walker, personal communication). No internal boundaries of this kind are present in any section of flow D41 that we have examined, suggesting that if any such intrusion occurred,

it was when the interior of the first unit of D41 was well above the solidus temperature. Thus most of the two units would still have cooled as one through the remanence blocking range. This conclusion is reinforced by microscopic examination of polished thin sections of samples in flow D41 for indications of relative cooling rate. The average maximum size of titanomagnetite crystals shows a simple trend, with its maximum in the central part (Fig. 4a). Moreover, the proportion of dendritic titanomagnetite, indicative of degree of undercooling, is maximal (near 100%) at the top and bottom boundaries and decreases progressively inwards.

Magnetic anisotropy. Preferred orientation of magnetic minerals and their easy axes of magnetization can deflect the direction of thermoremanence away from the ambient field^{11,12}. To cause the large deviations of remanence directions, however, this mineral fabric would have to be exceptionally strong in the interior and weakened progressively toward the boundaries. On the contrary, the fabric of flow D41 is extremely weak throughout and inconsistent in orientation: susceptibility anisotropy is small (<0.5%) and bears no relation to the angular deviation of remanence. Furthermore, thermoremanent magnetization imparted in the course of palaeointensity experiments⁵ to four samples of the anomalous flows from the original section deviated from the applied field by no more than a few degrees.

Viscous contamination. Our experience with Steens and other basaltic lavas is that the viscous overprint impressed by the present normal polarity Brunhes epoch is effectively eliminated

FIG. 3 a, Vector component diagrams illustrating how the direction of natural remanent magnetization (NRM) of samples from the interior of flow D41 (right) rotates from southerly toward westerly declinations during progressive thermal demagnetization above 300 °C, whereas that from samples near the margins (centre) and from lower flows (left) decay straight towards the origin with constant direction. Points are the projection of the remanence vector on the horizontal (open circles) and N-S vertical (filled circles) planes, and the numbers beside them are demagnetization temperatures in °C. Arrows denote unblocking temperature intervals used in the conductive cooling computation¹⁶ summarized in the text. b, Angular deviation of remanence after thermal demagnetization to 500 °C as a function of vertical position of samples in flows through the second directional jump (left); equal-area projections showing the full direction of samples from flows D41 and D40 after demagnetization to 500 °C (right). Angular deviation for each sample is the angle measured from the pre-jump direction (D42) toward the post-jump direction (A39) after projecting its direction onto the plane containing the flow D42 and A39 mean directions. Different symbols denote different vertical profiles within a horizontal distance of 10 m. Note the large swing in direction in flow D41 towards and away from A39 and the smaller such swing in flow D40.



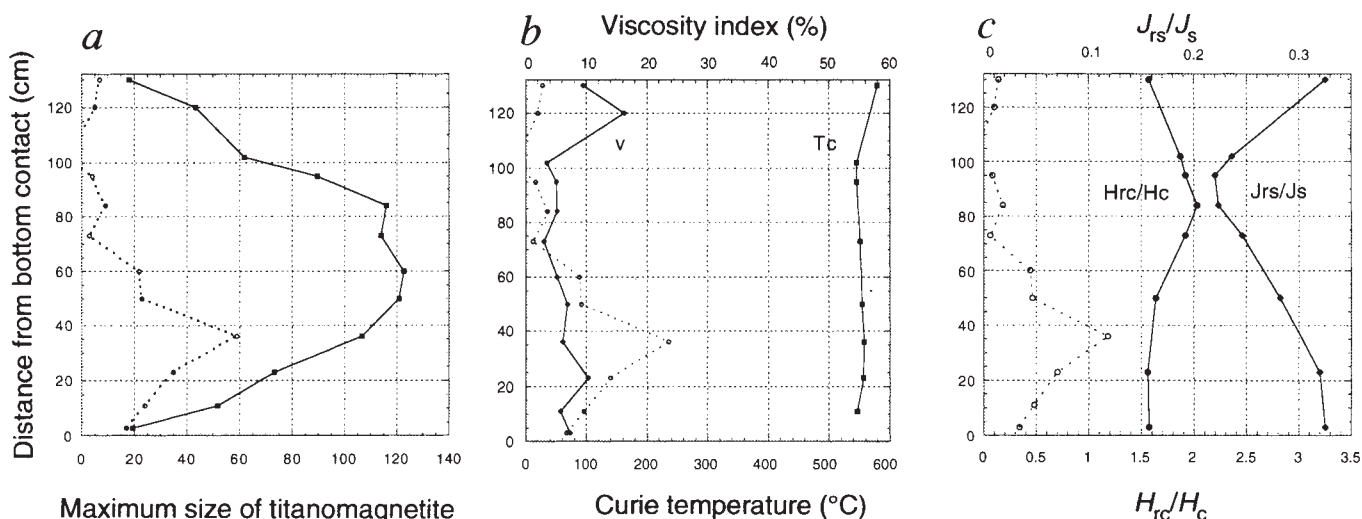


FIG. 4 Magnetic properties of samples (solid lines) through the critical flow D41 as a function of vertical distance above the bottom contact. Angular deviation of remanence (see Fig. 3b) also shown (dotted line) for reference. a, Average maximum grain size (μm) of titanomagnetite

crystals (not size of remanence-carrying magnetite particles—see text). b, Curie point T_c and viscosity coefficient V . c, Hysteresis parameter ratios indicative of magnetic grain size (J_{rs}/J_s = saturation remanence/saturation magnetization; H_{rc}/H_c = remanent coercivity/coercivity).

after demagnetization at temperatures ranging between 150 and 300 $^{\circ}\text{C}$. The overlying flow direction and that of the normal axial dipole field are far apart in the second jump, making it implausible that Brunhes viscous remanence could play any role in the observed pattern of directions in flow D41. To test the possibility that a more ancient unremoved viscous component (imparted by thermal enhancement of magnetic viscosity after the thick overlying flows A39 and A38 were emplaced) could explain the vertical distribution of remanence direction in flow D41, we measured the viscosity index, defined as the ratio of viscous remanence acquired in the ambient field in approximately two weeks to the stable natural remanent magnetization. It is low throughout the flow (generally <10%) and not correlated with the angular deviation of remanence (Fig. 4b), indicating once again that viscous remanence is not a viable explanation.

Chemical remanence. A similar but less easily discounted class of hypotheses is that a moderate rise in temperature or hydrothermal activity accompanying the emplacement of post-jump flows A39 and A38 might have caused some kind of partial chemical remagnetization of flow D41. Because chemical alteration can produce remanence with unblocking temperatures much higher than the temperature at which it occurs¹², a chemical remagnetization could have unblocking temperatures that strongly overlap those of the original thermoremanence. But as there is no *a priori* reason why such effects should be peaked at the centre of a 0.7-m zone of lava in the lower half of the flow rather than increasing steadily upwards or being concentrated along unit boundaries or cracks at the top and bottom, this interpretation needs to be supported by mineralogical observations or rock-magnetic data to be convincing.

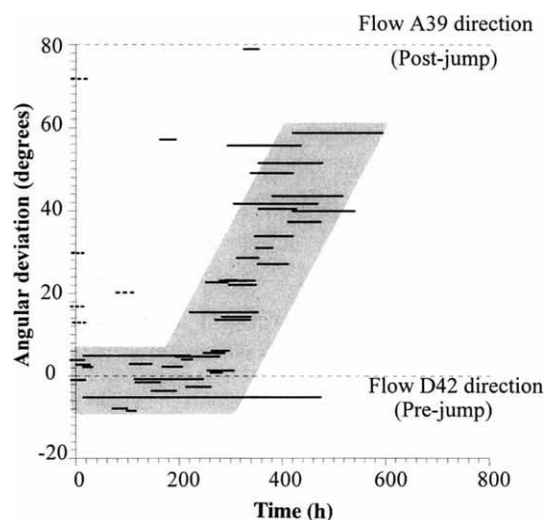
Despite considerable effort, we have found no such evidence to support this hypothesis. A detailed account of our observations and magnetic experiments will be published elsewhere, but we summarize here the most important results. In thin section under the petrographical microscope, flow D41 appears to be a common basalt, rich in plagioclase and olivine. Olivine is partially altered to iddingsite. Titanomagnetite grains are subdivided by lamellae of the usual trellis type, typical of deuteric oxidation (that is, oxidation during original cooling) at high temperature. Thermomagnetic curves in low field indicate single Curie points close to 555 $^{\circ}\text{C}$ and are reversible within 10–15%. This is consistent with the impression, gained by microscope

observations, of a simple magnetic mineralogy dominated by iron-rich titanomagnetite. There is no significant variation of Curie point with vertical position in the flow (Fig. 4b). About 5% of the natural remanence has blocking temperatures above 600 $^{\circ}\text{C}$, signalling the presence of titanohaematite, which is also a typical product of deuteric oxidation.

Magnetic hysteresis parameters should also provide clues whether magnetochemical changes occurred that could have caused the angular deviations in flow D41. In particular, the ratios of saturation remanence to saturation magnetization and remanent coercivity to coercivity (Fig. 4c) are very useful indicators of effective magnetic grain size. They demonstrate that the magnetic grains in flow D41 are pseudo-single-domain or interacting single-domain, not multidomain¹³. They are smallest at the top and bottom and reach a maximum 85 cm above the base, well above the anomalous angular deviations of direction and somewhat above the height at which the largest titanomagnetite grain size was observed microscopically. There is no contradiction with this latter observation, because the lamellae produced by the deuteric oxidation/exsolution process subdivide the titanomagnetite host grains. Thus the effective magnetic grain size is controlled by this process, rather than by the overall size of the titanomagnetite grain. It is important as well to emphasize that for magnetite grains like ours having J_{rs}/J_s (saturation remanence/saturation magnetization) ratios larger than 0.2, the blocking and unblocking temperatures of any partial thermoremanent magnetization (PTRM) are the same¹⁴. Even after allowance for the difference in rate of cooling in nature and rate of heating in the laboratory¹⁵, for such fine-grained particles the blocking temperature in nature of a given PTRM cannot be much larger than the unblocking temperature in the laboratory.

To sum up, we have failed to unearth any variation in composition, phase or grain size of magnetic minerals as a function of vertical position that would suggest that magnetochemical changes occurred preferentially in the lower third of flow D41 during the emplacement of post-jump flows A39 and A38. Although this negative result does not prove that the anomalous deviations in remanence directions we found there could not have been caused by some chemical mechanism, as yet unknown and for which we can find no clue, this is obviously not a satisfying explanation. Moreover, as described above, we did find both microscopic and magnetic evidence for magnetochemical alteration of the upper 60% of flow D40 by the overlying flow or

FIG. 5 Conductive cooling analysis by Camps *et al.*¹⁶ of the palaeomagnetic data reported here, assuming the rapid-field-change hypothesis. The analysis yields a rate of change of geomagnetic field of $6 \pm 2^\circ$ per day, as suggested by shading (see text). Angular deviation of direction obtained by thermal demagnetization of samples from flow D41, measured from the underlying (D42) flow direction toward the overlying (A39) flow direction, is plotted as a function of time elapsed since beginning of cooling (see text). Each bar indicates the time interval during which each direction of magnetization was blocked. Dashed bars correspond to samples near the flow top that were reheated by the overlying flow. (Figure adapted from ref. 16.)



flows. This alteration decreased in intensity downward, as would be expected, and did not affect the lower part of D40 or any of D41. Finally, we point out that flow B51 in the first directional gap, for which we reported earlier⁶ a similar pattern of angular deviation to D41 in the lower half (though not in the upper half because baking was much more pronounced—see Fig. 4 in ref. 6), has quite different magnetic properties from flow D41. Its magnetic mineralogy is more complex, with most samples exhibiting low-, intermediate- and high-temperature Curie points, and its magnetic grain size and magnetic viscosity are considerably larger. Therefore, it is unlikely that one rock-magnetic explanation could account for the vertical distribution of directions in both flows, whereas the single hypothesis that the geomagnetic field direction was changing extremely rapidly as they cooled can do so very naturally.

Conductive cooling calculations

For these reasons we return to the hypothesis of rapid field change during cooling to enquire whether it can explain quantitatively the thermal demagnetization results from flow D41. A calculation using the analytical error function solution⁹ for conductive cooling of a thin lava sheet emplaced instantaneously at $1,140^\circ\text{C}$ on a thick pile of already-cooled lava, and with its upper surface held at constant ambient temperature, predicts that the sample in flow D41 taking the longest time to cool to 500°C (about 6 days) would be the one which displays the largest angular deviation in Fig. 3b. This agrees with the rapid-field-change hypothesis. Numerical computation employing a more realistic model, which allows for temperature-dependent diffusivity, latent heat of crystallization released over a range of temperatures, and variable vesicularity with depth in flow D41, shows for a range of cases that the last sample to cool to 500°C would be either that with the largest angular deviation or the next lower sample, and that the cooling time would be between 6 and 16 days (ref. 16). Figure 5 shows a fit of this model to well defined remanent directions of PTRM in flow D41 for unblocking intervals above 300°C . One to four (on average, three) PTRMs from all samples in the three vertical profiles through the flow are included. The model assumes that as the flow cooled, each element of PTRM faithfully recorded the instantaneous field direction, and, as discussed above, that the blocking temperatures for each element are equal to their unblocking temperatures during thermal demagnetization. The time elapsed from when the flow began to cool until it cooled through the upper and lower blocking temperature of each PTRM is calculated and plotted against the angular deviation of PTRM direction. Thus the total magnetic recording interval plotted is the span of time over which various samples in flow

D41 were cooling through magnetic blocking temperatures between 680 and 300°C .

The general trend in Fig. 5 is consistent with a simple model in which the field remained stationary during the first half of the magnetic recording interval and then moved from the pre-jump towards the post-jump direction at a rate of $6 \pm 2^\circ$ per day. The fit to the data is reasonably good. Only seven PTRMs lie off the general trend. Five of those are shown as dashed bars in Fig. 5 because they are from samples in the upper 22 cm of the flow that were clearly remagnetized by baking, as discussed earlier, and so can be excluded. This is probably the case for the sixth, which is the lowest-temperature PTRM from the next sample down, still only 30 cm below the top. We have no explanation for the seventh outlier, which is the $525\text{--}575^\circ\text{C}$ PTRM from the sample deep within the flow that shows the peak in angular deviation in Fig. 3b.

There are two main aspects of our data that cannot be fitted by the idealized cooling models we have employed. The first, just mentioned, is the sharply peaked form of the angular deviation as a function of vertical position (Fig. 3b). We cannot dismiss it as an artefact due to a single outlying point because it is corroborated by two other samples from partially overlapping sections. The second aspect is the quantitative relationship of the angular deviations in the lower third of flow D40 to those in D41. The magnetic data suggest that D40 cooled quicker, and baked the top of D41 less, than the simple models predict. Previous workers have also noted the surprisingly small extent to which lava flows have reheated those underlying them¹⁷. This indicates that the assumption of cooling proceeding from an initially uniform temperature throughout the flow just after emplacement must be significantly incorrect. Such would be the case if appreciable heat were lost during the process of emplacement, as more sophisticated modelling indicates¹⁸, or if a later pulse of magma were injected into a previous flow unit erupted shortly before, as mentioned briefly above. Indeed, the slight changes in slope of the maximum-grain-size curve (Fig. 4a) around 25 and 100 cm above the base of flow D41 could conceivably be a result of such a two-stage emplacement process. These complications notwithstanding, the general compatibility of thermal demagnetization data for flow D41 with the simple conductive cooling model is an argument in support of the rapid-field-change hypothesis.

Broader implications of rapid field change

If this hypothesis is correct, it may imply that rapid jumps of field direction occur many times during reversal of polarity. If only three occurred during the reversal recorded at Steens Mountain, corresponding to the three gaps labelled in Fig. 1, and if it

took several thousand years to complete, as believed to be the cases for this reversal³ and for reversals in general^{19, 20}, the probability of one of the 56 transitional lava flows cooling through its magnetic blocking range during one of these jumps is a few tenths of a per cent. The Steens Mountain section records two such occurrences. Thus it would either have to be an exceptionally rare recording, or many other rapid changes of field direction occurred that were not recorded. Perhaps there were extended intervals when the field jumped repeatedly, either randomly or in an oscillatory manner, or perhaps many isolated jumps occurred. Because of the fragmentary nature of lava flow records and the decade or longer time resolution of sedimentary records, these possibilities are difficult to prove or disprove.

Whether such rapid jumps of the geomagnetic field might be generated externally is an open question. It has been suggested that during the reversal recorded at Steens Mountain, when the internally generated field was weak, enhanced external field activity due, for example, to greater penetration of charged particles from the Sun²¹ might somehow cause the jumps. Although attractive because of their ability to produce rapid field change, a difficulty inherent in all such external mechanisms is that the fast swing in direction occurred between relatively stable beginning and ending directions. In other words, at the second directional gap, the field must have maintained the pre- and post-jump directions for several tens of weeks at a minimum, enough time for two flows spanning 28 m and five flows spanning 18 m (Fig. 2) to be erupted and cool. It is hard to understand how such long-lived directions anchored to the Earth could be produced by the external field, and even harder to imagine how the external and internal fields could work in concert to produce the jump in direction.

On the other hand, one must ask whether magnetic screening due to the electrical conductivity of the mantle would permit such a rapid field change to be observed at the surface if it were generated internally. The conductivity of rocks near the surface is so low that their screening is negligible, but electromagnetic induction studies have long suggested that conductivity increases significantly with depth in the mantle^{22, 23}. Several recent studies suggest that this increase may occur at the phase transition at 660 km depth, reaching an average value of the order of 1 S m^{-1} in the lower mantle^{24–27}, which is lower by a factor of 10–1,000 than believed earlier. Laboratory measurements of conductivity

of presumed lower-mantle minerals at high pressure and temperature are consistent with these low estimates²⁸ or are even lower^{29, 30}. For such a mantle conductivity profile (0 S m^{-1} above 660 km depth, and 1 S m^{-1} below), we calculate that the characteristic smoothing time^{31, 32} for an impulsive change in magnetic field of spherical harmonic degree 1 and 6 at the core-mantle boundary is 20 and 6 days, respectively. We consider that a degree of more than one is more realistic because such rapid changes at greatly diminished field intensity suggest a local, non-dipolar source. Thus the rates of change inferred from our data are not incompatible with present estimates of mantle conductivity. Moreover, it is widely held that mantle conductivity is laterally heterogeneous on a large scale^{33–37}, which would allow such rapid signals to be observed at the Earth's surface above regions of low conductivity even if the average were considerably greater than 1 S m^{-1} .

Concluding remarks

The large-amplitude directional deviations of remanent magnetization within single lava flows reported here, and their unusual dependence on both vertical position and unblocking temperature, are unique in our experience. Their occurrence in two flows at two different locations and stratigraphic positions where large jumps occur in the Steens Mountain record is probably not coincidental. Putting aside for the moment that field changes 1,000 times more rapid than observed for the modern field are implied, the hypothesis that the reversing field changed several tens of degrees in direction during the cooling of these flows is by far the most natural and satisfying explanation of our observations. Even though such extraordinary rates of change are inescapable under this hypothesis, it still seems preferable to any rock-magnetic explanation that we can imagine, each of which proves to be severely contrived when examined closely and tested with measurements of magnetic properties.

This is not to suppose that geomagnetic reversals take place much more quickly than the several thousand years currently supposed, but rather to suggest that polarity transitions may be punctuated by episodes of extraordinarily rapid field change. An internal origin is not excluded by recent estimates of mantle conductivity, whereas an external mechanism cannot easily account for the relatively long-standing pre- and post-jump directions. Thus such rapid signals may well originate in the outermost core. □

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The structure of *trp* RNA-binding attenuation protein

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The crystal structure of the *trp* RNA-binding attenuation protein of *Bacillus subtilis* solved at 1.8 Å resolution reveals a novel structural arrangement in which the eleven subunits are stabilized through eleven intersubunit β -sheets to form a β -wheel with a large central hole. The nature of the binding of L-tryptophan in clefts between adjacent β -sheets in the β -wheel suggests that this binding induces conformational changes in the flexible residues 25–33 and 49–52. It is argued that upon binding, the messenger RNA target forms a matching circle in which eleven U/GAG repeats are bound to the surface of the protein ondecamer modified by the binding of L-tryptophan.

EXPRESSION of the tryptophan (*trpEDCFBA*) operon of *Bacillus subtilis* is negatively regulated by the *trp* RNA-binding attenuation protein (TRAP) in response to intracellular levels of L-tryptophan¹. Transcription of the operon is attenuated by a mechanism that functions in the 204-nucleotide leader region preceding the structural genes of the operon². When activated by L-tryptophan, TRAP binds to the leader transcript RNA, inducing formation of a transcription terminator structure, and the expression of the operon is halted^{3,4}. In the absence of L-tryptophan, TRAP does not bind to the leader RNA, an alternative RNA structure forms the antiterminator and the operon is expressed. TRAP has also been implicated in regulating translation of two *trp* genes, *trpE* and *trpG*, by two different mechanisms. It was suggested that TRAP binding to the leader region of *trp* transcript induces formation of another RNA secondary structure⁵ (different from those described above) which sequesters the ribosome-binding site for *trpE*, the first structural gene of the operon. For *trpG*, which is not located within the *trp* operon⁶, TRAP has been suggested to compete directly with the ribosome for binding to the region immediately preceding the start of *trpG*, and thus to repress translation of this gene in response to L-tryptophan.

Similar regulatory mechanisms involving RNA-binding proteins have been described for other bacterial systems^{7–10}. This attenuation mechanism contrasts with that which regulates the *trp* operon of *Escherichia coli* and several other enteric bacteria. In these bacteria, the ability of a ribosome to translate tandem *trp* codons in a leader peptide controls the leader transcript's secondary structure¹¹. Attenuation controls expression of several eukaryotic genes¹², but it is not yet clear whether RNA-binding proteins such as TRAP are involved in the mechanism.

Very little is known about the nature of proteins that bind mRNA specifically in important cellular processes such as transcription, translation and RNA processing. However, several conserved motifs have been found in RNA-binding proteins involved in post-transcriptional regulation of gene expression. Some of these motifs are directly involved in RNA binding¹³. These include the RNP, arginine-rich, RGG box and KH motifs. Detailed structural information is so far available only for the RNP motif. The crystal structure of U1A spliceosomal protein¹⁴, which contains the RNP motif, shows that an RNA hairpin binds to the central two β -strands of the 4-stranded antiparallel

β -sheet and to flexible loops on one end of the β -sheet. TRAP does not contain any of the known RNA-binding sequence motifs and the crystal structures show that it has a polypeptide-chain fold different from that of U1A. The TRAP fold is unrelated to any structure in the Protein Data Bank¹⁵.

The available evidence suggests TRAP binds single-stranded RNA. Several attempts have been made to determine the specific TRAP binding site in the *trp* leader RNA^{5,16}. It has been suggested¹⁶ that TRAP recognizes GAG and UAG nucleotide motifs, which are repeated eleven times in the *trp* leader transcript and nine times in the *trpG* leader transcript.

TRAP is encoded by the *mrpB* gene of *B. subtilis*¹⁷. There are no tryptophans, cysteines or prolines in this protein. Sequence comparison has not revealed any similar proteins, although there is a 20% sequence identity with RegA¹⁸, an RNA-binding translational repressor from bacteriophage T4, the three-dimensional structure of which is unknown. TRAP functions as an oligomer^{3,4,16} of eleven identical subunits¹⁹. Here we report the X-ray structure of TRAP refined at 1.8 Å resolution, defining the complete three-dimensional structure of the 11-mer in its L-tryptophan-activated form. This oligomeric protein consists of eleven β -sheets arranged in a ring and defines a new tertiary fold which we refer to as a β -wheel. From our results, we can infer the mechanism of activation of TRAP by L-tryptophan and the possible nature of the interaction with the *trp* leader transcript RNA.

Structure determination and refinement

TRAP was crystallized in the presence of either L-tryptophan or 5-Br-L-tryptophan¹⁹. The asymmetric unit of the crystals, which belong to the space group C2, with $a = 157.1$ Å, $b = 114.7$ Å, $c = 106.3$ Å, $\beta = 117.7^\circ$, contains two eleven-subunit oligomers, each with an internal 11-fold axis of symmetry, lying face to face. The structure was solved by single isomorphous replacement with the use of anomalous scattering. The 22 bromine positions were located by a molecular replacement search against isomorphous and anomalous differences using an 11-atom ring as a search model. The initial electron density was improved by 22-fold averaging, which was alternated with cycles of phase extension from 2.9 to 2.2 Å. Refinement statistics are shown in Table 1 together with further details of the X-ray analysis. The structure was refined to a crystallographic *R*-factor of 0.176

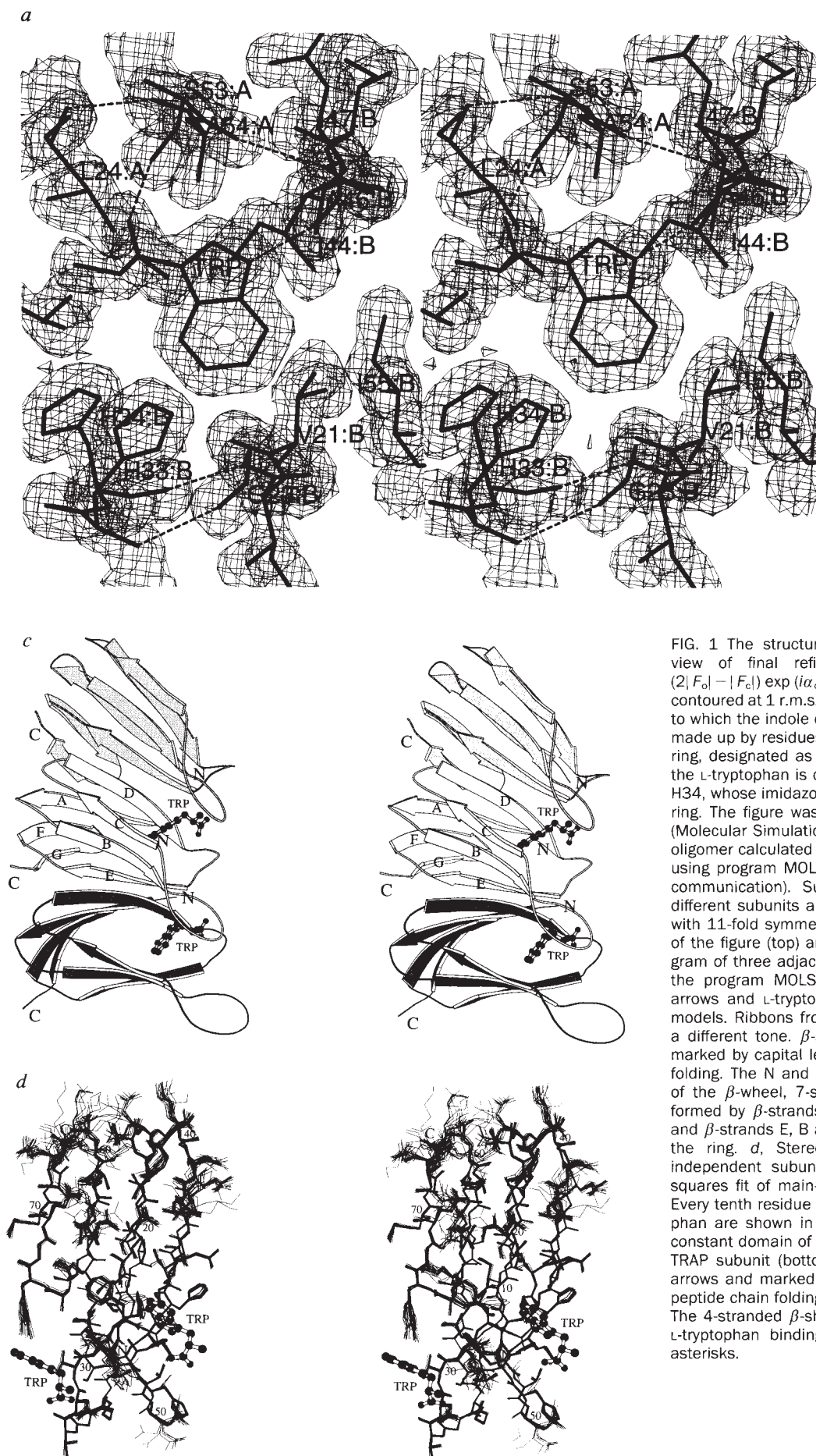
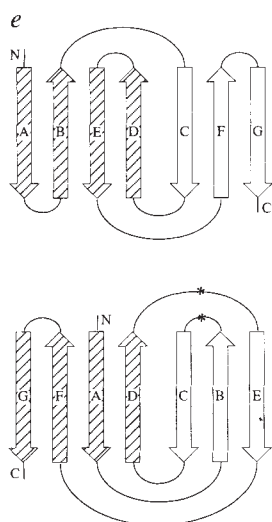
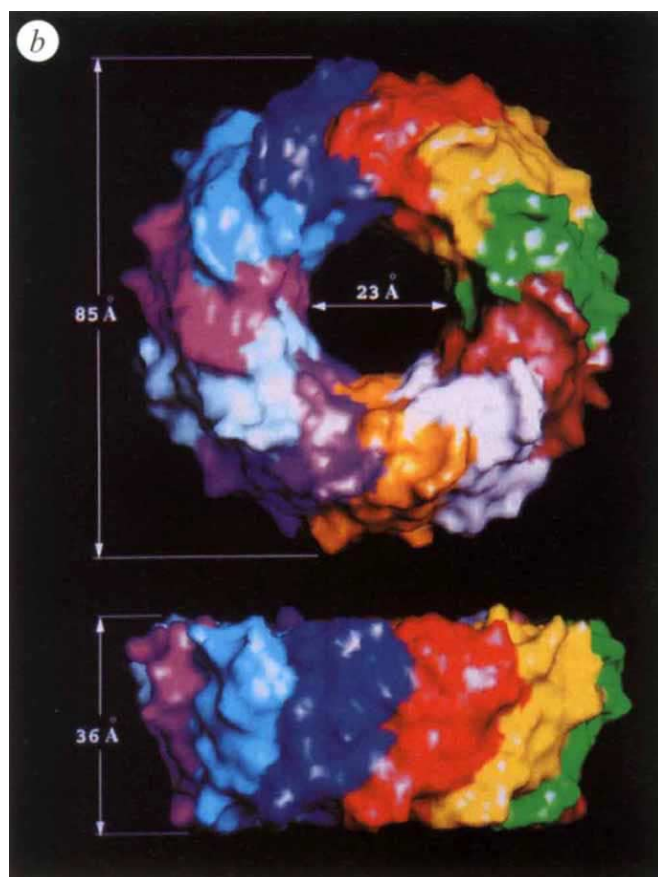


FIG. 1 The structural features of TRAP. **a**, A stereo view of final refined electron density of TRAP, $(2|F_o| - |F_c|) \exp(i\alpha_c)$, calculated at 1.8 Å resolution and contoured at 1 r.m.s. This shows the hydrophobic cluster to which the indole of L-tryptophan binds. The pocket is made up by residues from two adjacent subunits in the ring, designated as A and B. At the molecular surface the L-tryptophan is covered by two histidines, H33 and H34, whose imidazole carbons point towards the indole ring. The figure was drawn with the QUANTA program (Molecular Simulations). **b**, Molecular surface of whole oligomer calculated with a probe sphere radius of 1.5 Å using program MOLVIEWER (M. J. Hartshorn, personal communication). Surfaces corresponding to the 11 different subunits are shown in different colours. View with 11-fold symmetry axis perpendicular to the plane of the figure (top) and vertical (bottom). **c**, Ribbon diagram of three adjacent subunits of TRAP generated by the program MOLSCRIPT³⁵. β -strands are shown as arrows and L-tryptophan molecules as ball and stick models. Ribbons from different subunits are shown in a different tone. β -strands of the middle subunit are marked by capital letters in order of polypeptide-chain folding. The N and C termini are labelled. The 'wings' of the β -wheel, 7-stranded antiparallel β -sheets, are formed by β -strands G, F, A and D from one subunit, and β -strands E, B and C from the adjacent subunit in the ring. **d**, Stereo plot of 22 crystallographically independent subunits of TRAP overlapped by least-squares fit of main-chain atoms of residues 9 to 73. Every tenth residue is numbered. Molecules of L-tryptophan are shown in ball and stick. **e**, Topology of the constant domain of immunoglobulins²² (top) versus the TRAP subunit (bottom). β -strands are represented by arrows and marked by capital letters in order of polypeptide chain folding. The N and C termini are labelled. The 4-stranded β -sheet is highlighted by shading. The L-tryptophan binding loops of TRAP are labelled by asterisks.



($R_{\text{free}} = 0.225$) at 1.8 Å resolution, using synchrotron radiation X-ray data collected at cryotemperatures. Figure 1a shows the final electron density.

Structure description

The TRAP molecule contains eleven identical subunits arranged with almost exact symmetry in a ring to form a doughnut-like structure (Fig. 1b). TRAP belongs to the β -class of proteins²⁰, with 56% of its residues arranged in β -strands. The core of the oligomer is formed by a β -wheel consisting of eleven segments, each of which is a 7-stranded antiparallel β -sheet made up by β -strands from two adjacent subunits in the ring (Fig. 1c). Every subunit contributes three β -strands to one β -sheet and the other

four β -strands to the adjacent β -sheet in the ring. The atomic structure is essentially the same in each of the 22 crystallographically independent subunits, although side chain conformations of some residues on the oligomer surface vary considerably (Fig. 1d).

A search of the Protein Data Bank¹⁵ by the program SQUID²¹ did not reveal any protein with a polypeptide-chain fold like that of TRAP, but did indicate that, as in the immunoglobulins²², the polypeptide chain folds into 3-stranded and 4-stranded antiparallel β -sheets, facing each other. However, this resemblance of the polypeptide-chain folds is superficial as the connectivity between the β -strands is different (Fig. 1e).

The quaternary structure of the oligomer is maintained by hydrogen bonds formed between D and E β -strands from adjacent subunits in the ring. On both sides of this polar interface there are hydrophobic interactions (Fig. 2a). In addition, the bound molecule of L-tryptophan stabilizes the quaternary structure by hydrogen bonds and hydrophobic interactions with both adjacent subunits in the ring.

L-tryptophan binding

Each of the eleven L-tryptophan molecules binds between adjacent β -sheets of the β -wheel. The indole ring of L-tryptophan is buried in a hydrophobic pocket made up by residues from two adjacent subunits (Fig. 1a). The indole ring nitrogen is hydrogen-bonded with the carbonyl oxygen of Q47. The amino and carboxyl moieties of the L-tryptophan are bound to two adjacent subunits of TRAP through eight hydrogen bonds (Fig. 2b). Two of these are made with residues 49, 52 from one subunit and six with residues 25, 27, 29, 30, 53 from the adjacent subunit. In addition, one of the carboxyl oxygens is hydrogen-bonded to a water molecule. The two loops formed by residues 25–33 and 49–52 make no hydrogen bonds or hydrophobic interactions with the rest of the protein; their conformation is maintained by the bound molecule of L-tryptophan.

As a result of the extensive interactions with amino acids from two adjacent subunits, the bound L-tryptophan is almost completely buried and inaccessible to solvent, with only a single water molecule being coordinated to its carboxyl group. This mode of binding requires that in the absence of L-tryptophan, this portion of TRAP has an alternative conformation which allows the L-tryptophan molecule to enter the binding site. The binding would proceed through insertion of the indole into the hydrophobic pocket, with subsequent closing of the 'gate' by the two loops that wrap around the amino and carboxyl group of the L-tryptophan.

Adjacent molecules of L-tryptophan are relatively close to each other, with an average distance of 14.6 Å between Ca -atoms. Equilibrium dialysis experiments show that TRAP binds one molecule of L-tryptophan per subunit in a highly cooperative manner (Fig. 2c). This cooperativity may be explained by the interaction of the beginning of loop 46–54 with one L-tryptophan and by the end of this loop with the next L-tryptophan in the ring. Likewise, the end of the loop 24–34 interacts with one L-tryptophan and the beginning of this loop interacts with the next L-tryptophan in the ring. Thus binding of L-tryptophan in one cleft may affect the conformation of residues in the next cleft, and, as follows from equilibrium dialysis experiments, promote binding of L-tryptophan there.

The apparent molecular weight of TRAP is the same in the presence or absence of L-tryptophan^{3,4,16}, indicating that the number of subunits is the same in active and inactive TRAP. But the loops formed by residues 25–33 and 49–52 may well be flexible in the absence of L-tryptophan or have another conformation, in which the L-tryptophan binding clefts are accessible. This idea is consistent with the circular dichroism spectrum, which indicates that part of the structure has a different conformation in the absence of L-tryptophan (C. T. Baumann, personal communication).

The binding site of L-tryptophan in TRAP differs from that in the *trp* repressor^{23,24}. The hydrophobic cleft in TRAP involves more residues than in the *trp* repressor, where it is made up largely of the aliphatic arms of two arginines. In TRAP, the nitrogen atom of the indole ring is hydrogen-bonded to protein, unlike in *trp* repressor. However, there are some similarities in the nature of L-tryptophan binding: in both proteins the L-tryptophan binding involves amino-acid residues from two subunits and in neither protein do any aromatic side chains interact with bound L-tryptophan.

Mutants

We have also characterized six missense mutations in *mtrB* that result in loss of regulation of the *trp* operon (Fig. 4a). Most of these are negative dominant mutations, suggesting that the mutant peptides fold appropriately. The structure shows that mutants G23D (in which glycine at residue 23 is substituted by aspartate) and I44N (ref. 17) place charged or polar side chains into the hydrophobic pocket near the indole ring, where they prevent L-tryptophan binding. L24P (ref. 17) and F48L TRAP do not bind L-tryptophan or *trp* leader RNA *in vitro* owing to

disruption of the local environment near the L-tryptophan indole. E42 is at the dimer interface, situated close to K56 and R58 from the adjacent subunit, to which it makes a salt bridge in some of the 22 crystallographically independent subunits. Substitution of this residue with lysine (E42K) may result in electrostatic repulsion of adjacent subunits and thus prevent oligomerization. In the L38F (ref. 17) mutant, insertion of a more bulky phenylalanine instead of a lysine residue at this position would disrupt the hydrophobic core between the two β -sheets and induce conformational changes that affect oligomerization.

RNA target

The TRAP recognition site in the *trp* leader RNA was first proposed to consist of two 10-base direct repeats beginning at positions +37 and +69 (ref. 5; labelled DR1 and DR2 in Fig. 3a, b). It has been proposed that TRAP specifically binds eleven trinucleotides located between positions +36 and +91 of the *trp* leader transcript, of which seven are GAG and four UAG, each separated by two or three nucleotides¹⁶.

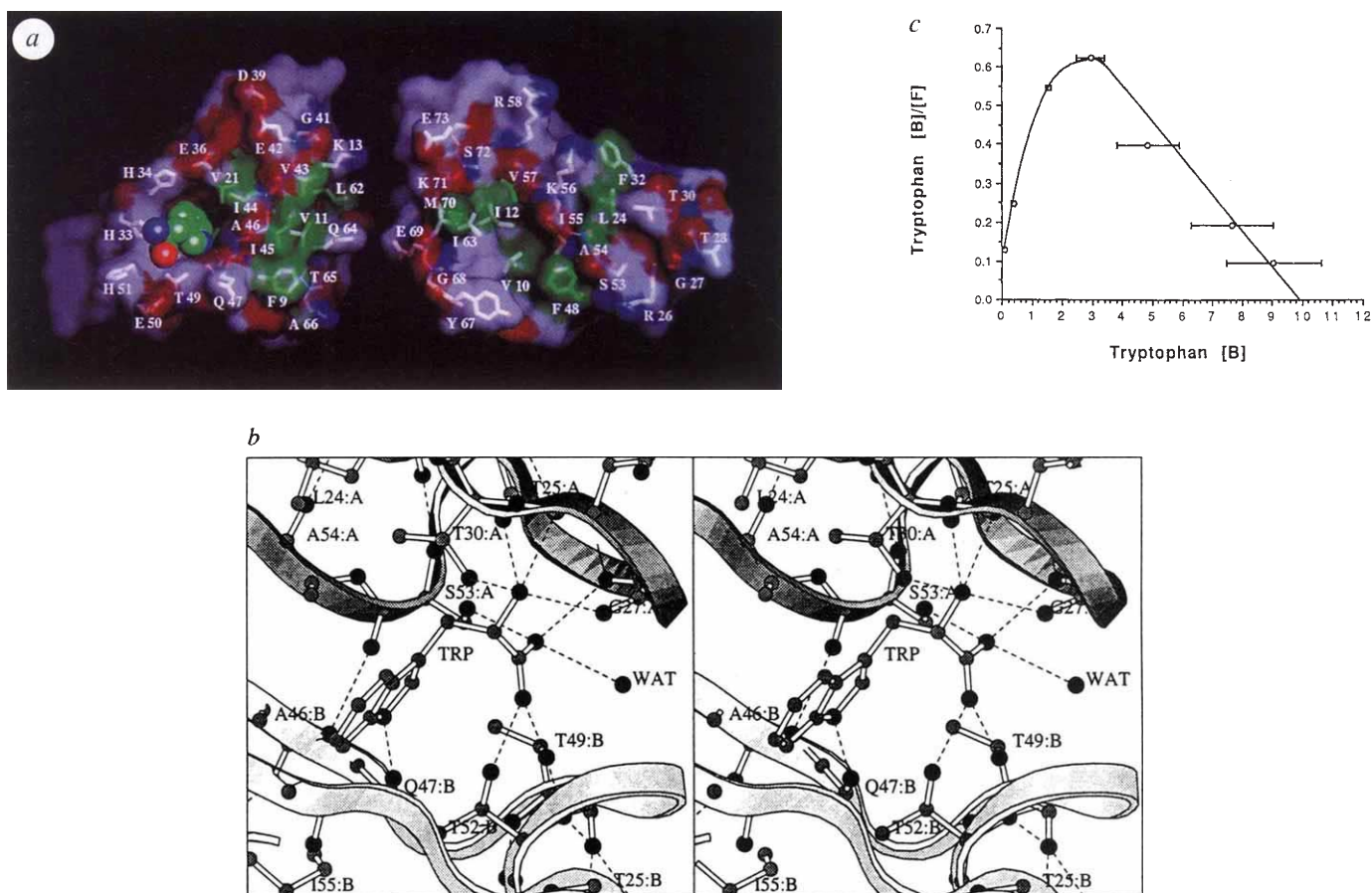


FIG. 2 L-tryptophan binding. *a*, Two adjacent subunits are separated and rotated by 90 degrees (left subunit clockwise and right subunit anticlockwise) to show the intersubunit contact surfaces. L-tryptophan is shown as a van der Waals model, with carboxyl oxygens in red, nitrogens in blue and carbons in green. The view is with the 11-fold symmetry axis in horizontal plane. Molecular surfaces are calculated with a probe sphere radius of 1.5 Å. The surface is coloured according to the properties of the nearest atoms, with negative charges shown in red, positive charges in blue, hydrophobic residues in green and others in grey. Residues forming the interface are shown as stick models. Surface around atoms lying further than 4.5 Å from the subunit interface is in grey. *b*, Network of hydrogen-bond interactions between L-tryptophan and TRAP. Ball-and-stick plot with residues from two adjacent subunits designated as A and B. Ribbons drawn through the main-chain atoms of two adjacent subunits are shown in light and dark tone. The

amino group of L-tryptophan is hydrogen-bonded to the peptide carbonyls of residues 25, 27, 29 and to the side-chain hydroxyl of T30. One carboxyl oxygen from L-tryptophan forms hydrogen bonds with the peptide amide of G27, the hydroxyl group of S53 and with a water molecule. The other carboxyl oxygen makes two hydrogen bonds with the hydroxyl groups of T49 and T52. Panels *a* and *b* were drawn by the program MOLVIEWER (M. J. Hartshorn, personal communication). *c*, Scatchard plot of L-tryptophan binding from equilibrium dialysis experiments using radiolabelled L-[¹⁴C]tryptophan (Dupont/NEN) and purified TRAP in lysate buffer (100 mM K₂HPO₄, pH 7.4, 50 mM KCl, 1 mM EDTA, 10% glycerol). Assays were done in a Hoefer Scientific equilibrium dialysis apparatus (model EMD101) with 1–4 μM TRAP (*M*, 91,600) and varying concentrations of L-[¹⁴C]tryptophan between 1–100 μM. After incubation at 5 °C overnight, aliquots were removed for liquid scintillation counting.

To characterize further the RNA target of TRAP, we tested a series of 5' and 3' deletions of the *trp* leader (Fig. 3a) using an *in vivo* titration assay⁵. Removal of up to the first 49 nucleotides has little effect on the ability of *trp* leader RNAs to titrate TRAP away from the reporter *trpE'*-*lacZ* fusion. Further deletions reduced the ability of the resulting RNAs to compete for TRAP until almost no titration activity was observed for the RNA encompassing nucleotides 66–138 (RNA 66–138). Deletions from the 3' end showed a similar pattern of effects. Our results confirm that the previously proposed 10-base direct repeats (DR1 and DR2) are not solely responsible for TRAP recognition. RNA 49–138, which lacks DR1, titrates TRAP nearly as well as the entire leader, but RNAs 57–138 and 62–138, which contain DR2, had little titration activity. Moreover, deletions outside DR1 or DR2 have significant effects on TRAP titration. There is, however, a correlation between the number of G/UAG repeats and ability to titrate TRAP. RNAs with 8–11 repeats titrate well, those with 6 or 7 repeats have moderate activity, but almost no TRAP-titrating ability was seen with RNAs containing 5 repeats.

To identify the role of each particular nucleotide in TRAP recognition, we made site-directed substitutions (Fig. 3b). Altering the third position of either three or five repeats from G to A (RNAs I and II) decreased TRAP titration accordingly. Similar results were seen when the second position of repeats 3–7 were altered (RNA III). Changing the first position of these repeats, however, had a significantly smaller effect (RNA IV). These results further support the G/UAG model for the TRAP binding

site and indicate that the last two residues of the repeats are more important for TRAP recognition than the first position.

Although the identities of the nucleotides between repeated elements in the *trp* leader are not conserved, the repeats are always separated by 2 or 3 residues. RNAs with only one residue (RNA V) or no residues between all eleven repeats (RNA VI) did not titrate TRAP, confirming that spacing of the repeats is important. But, RNAs with altered spacing between the first six, last six or middle six repeats (RNAs VII, VIII, IX) titrate TRAP well. Each of these RNAs contains at least six properly spaced repeats and several others spaced by five residues. There is no appreciable bias as to which repeats must be correctly spaced because RNAs VII and VIII titrate TRAP equally well. TRAP recognition therefore not only requires multiple G/UAG repeats, but these repeats must be properly spaced by at least two nucleotides.

Functional implications

In Fig. 4a the sequences are shown of TRAP from two closely related bacteria, *B. subtilis* and *B. pumilus*. The two sequences are highly homologous, with sequence identity of 77%. From these two sequences alone it is not possible to identify directly the functionally important residues, but some conclusions can be drawn. There are three sets of residues which need to be conserved: (1) those in the hydrophobic interior; (2) those involved in the intersubunit interface; and (3) those directly involved in tryptophan binding. But an additional set is also conserved on the surface of the TRAP doughnut containing

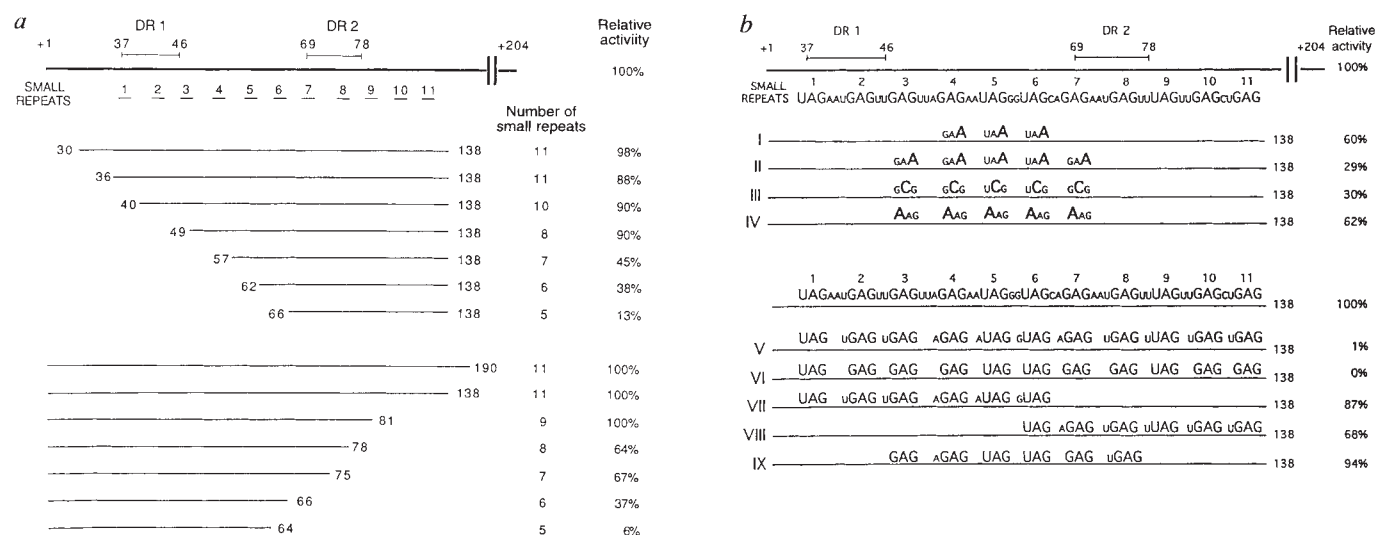


FIG. 3 Analysis of the TRAP RNA target. The schematic diagrams represent the *trp* leader transcript with the relative positions of the 10-base direct repeats, DR1 and DR2, and the 11 small repeats, U/GAG, numbered sequentially. The relative TRAP-titrating activities of modified *trp* leader segments are shown based on comparison to the full-length *trp* leader, positions +1 to +204, which yields 1,500 Miller units, set at 100%. Each value is based on the average of two or more experiments done in triplicate. Independent measurements with the same RNA segment differed by no more than 5%. Predictions of secondary structures for all modified RNAs performed using program GCG³⁶ showed that blocking hairpin structures, which may affect binding to TRAP, are no more likely to form than in wild-type full-length sequence. a, 5' and 3' deletions of the *trp* leader containing various numbers of U/GAG repeats. b, Site-directed mutants of *trp* leader section +1 to +138. The sequence between +36 and +91 is shown, with 11 U/GAG small repeats in large type. On RNAs I to IV only mutated repeats are indicated, with the altered base in bold large type. For RNAs V to IX only repeats adjacent to deleted spacer residues are shown, with gaps between repeats representing deleted spacer residues.

METHODS. The *in vivo* titration assay of Kuroda *et al.*⁵ was used to measure the ability of overexpressed segments of the *trp* leader RNA

to titrate TRAP and lead to deregulated overexpression of a single copy *trpE'*-*lacZ* translational fusion under control of the *trp* promoter and leader region. To create plasmids to overexpress the *trp* leader RNA, the *trp* promoter was subcloned into pMK104 (ref. 5) on an *EcoRI*-*XbaI* fragment after inserting a C between +4 and +5 of the *trp* leader to create an *XbaI* site. The *EcoRI* site was subsequently destroyed and the *XbaI* to *HindIII* polylinker region was replaced with the polylinker of pUC18 to create pJ1. For deletions, sections of the *trp* leader were amplified using 5' primers incorporating an *EcoRI* or *XbaI* site and 3' primers with a *HindIII* site and subcloned into pJ1. For site-directed mutations, the +4 to +138 *trp* leader region was used as a template for oligonucleotide-directed *in vitro* mutagenesis (Amersham Sculptor kit) and then subcloned into pJ1. *B. subtilis* strain CYBS 12 (ref. 5) was transformed with the pJ1 constructs and assayed for β -galactosidase activity³⁷. The *in vivo* steady-state levels of several of the competing RNAs were tested by dot-blot hybridization using an antisense RNA probe complementary to the region between positions +36 and +81 of the *trp* leader transcript. The levels were found to be similar for several of the 5' and 3' deletion RNAs except internal deletion RNAs 36–81 and 40–81 (not shown on the figure), which had significantly lower steady-state levels and had little titration activity.

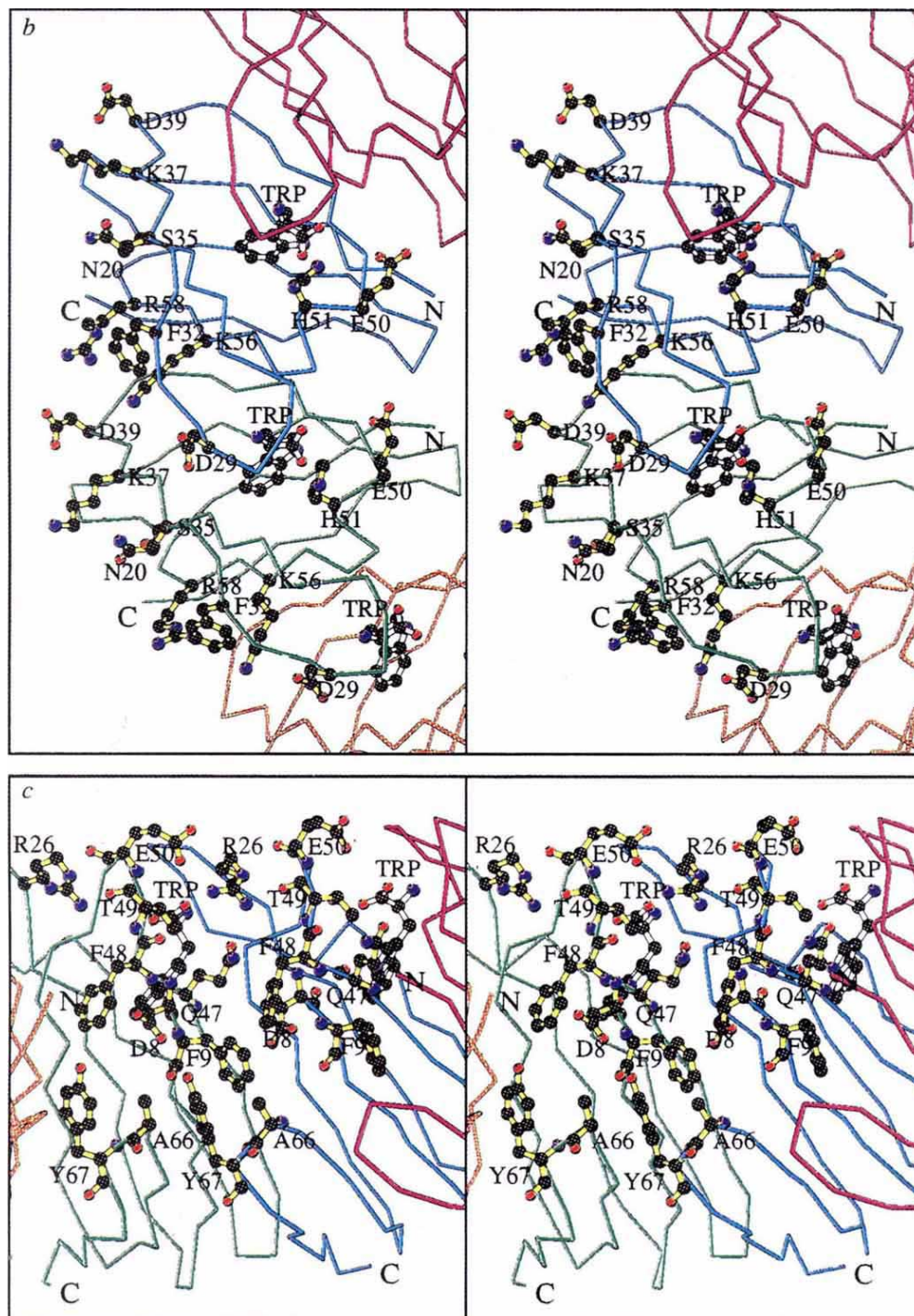
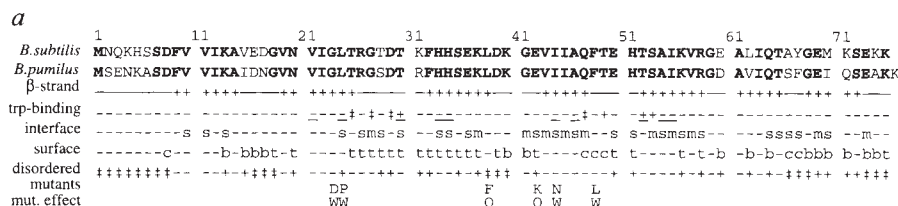


FIG. 4 Functionally important residues of TRAP from *B. subtilis*¹⁷ and *B. pumilus*³⁸. **a**, Alignment of sequences of TRAP from *B. subtilis*¹⁷ and *B. pumilus*³⁸. Key to characters in lines: β -strand, residues lying in β -strands are highlighted by +; trp-binding, L-tryptophan-binding residues are designated by + for hydrogen bonding by side chain and ‡ for hydrogen bonding by main chain; interface, residues within 3.5 Å from the adjacent subunit in the ring, are designated 's' for side-chain intersubunit interactions and 'm' for main-chain intersubunit contacts; surface, solvent-accessible residues are designated 't' for the top molecular surface (Fig. 1b, top), 'b' for the opposite surface of the doughnut, and 'c' for the surface of the oligomer channel; disordered, r.m.s. deviation from mean value calculated using 22 crystallographically independent subunits ('+' indicates only side-chain atoms with r.m.s. greater than 0.04 Å; '‡' main-chain atoms with r.m.s. greater than 0.04 Å); mutation effect, W affects L-tryptophan binding, O affects subunit assembly. **b, c** Surface composition of TRAP generated by the program MOLSCRIPT³⁵. Four adjacent subunits in the ring are represented as a ribbons of different colour. Ball-and-stick models are shown only for the central pair of subunits. **b**, Surface at which L-tryptophan binds. Only surface residues that are conserved in the two sequences of TRAP from different bacterial sources and are not involved in intersubunit contacts are shown. **c**, Surface residues of the inner cavity of TRAP viewed from the centre of the doughnut. The diameter of the cylinder which could fit into the van der Waals model is smaller at the bottom of the cavity where it is 22.8 Å. The cavity is larger at the top, about 35.0 Å, as defined by the guanidinium groups of R26. The diameter of the TRAP channel could be affected by the seven N-terminal residues as the first clearly defined amino acid, D8, is situated in the middle part of the cavity surface with its amide pointing toward the top of the cavity. Modelling of the 7 N-terminal amino acids into weak electron density shows that they probably do not decrease the overall diameter of the cavity. The first 6 amino acids vary considerably between TRAP from *B. subtilis* and *B. pumilus* and our studies using a mutant lacking residues 2–6 indicate these are not essential for TRAP function.

residues that are considered to undergo a conformational change upon L-tryptophan binding (Fig. 4b). This set includes residues D29, F32, E50 and H51 in the two loops whose buried amino acids interact with L-tryptophan, and the nearby residues N20, S35, K37, D39, K56 and R58. These residues have no obvious structural role in the native protein; indeed, they all have poorly ordered side chains, with an r.m.s. deviation from mean value of greater than 0.04 Å (Fig. 4a). There is only one conserved disordered residue on the opposite surface of the doughnut (K40) and one inside the channel (D8). This allows us to identify the conserved residues on the L-tryptophan binding surface as potential candidates for RNA binding.

The 11-fold symmetry of the TRAP oligomer suggests that upon binding the RNA 56-mer containing the eleven GAG/UAG elements should form a matching circle to present all eleven repeats to the binding surfaces of the TRAP molecule (Fig. 5). The RNA circle may bind between two molecules of TRAP in a sandwich-type structure. Site-directed substitutions indicate that the spacers between the U/GAG repeats should be at least two nucleotides long. Our study of transfer RNA coordinates²⁵ shows that the phosphate-phosphate spacing between nucleotides n and $n+5$ along the RNA backbone can vary between 8.5 and 29.2 Å, with an average distance of 20.2 Å. This allows the radius of the RNA circle that has eleven 5-

TABLE 1 Data collection and refinement statistics

Data collection			
Resolution range	10.0–1.8 Å		
Number of unique reflections	147,134		
Redundancy*	3.69		
Completeness	97.5%		
Reflections with $I > 3\sigma_I$	72.1%		
$R_{\text{merge}}^{\dagger}$	0.069		
Refinement and model correlation			
Number of atoms	13,852	Deviations from ideal geometry [‡]	
Number of reflections used in refinement	145,663	Bond distance (Å)	0.020 (0.020)
R -factor [‡]	0.178	Angle distance (Å)	0.039 (0.035)
Number of reflections used for R_{free}	1,471	Planes (Å)	0.008 (0.020)
$R_{\text{free}}^{\ddagger}$	0.225	Non-crystallographic symmetry (Å) [#]	0.053 (main chain)
Real-space fit [§]	0.979 (main chain)		0.218 (side chain)
	0.918 (side chain)		
Average atomic B-factor (Å ²)	20.3		

Initial X-ray data from complexes of TRAP with L-tryptophan and with 5-Br-L-tryptophan were collected at the EMBL Hamburg beamlines as described¹⁹. The asymmetric unit of the crystal contains two ondecamers and as each subunit binds one L-tryptophan molecule, we expected to find 22 Br atoms per asymmetric part of the crystal. Different methods were tried to find the heavy atoms. Neither the isomorphous nor the anomalous difference Patterson showed a clear vector pattern. Automatic Patterson search and direct methods failed to find a solution. To find the 22 Br positions, we used a new procedure. 11-fold symmetry requires that identical atoms in the 11 subunits of the ondecamer should lie in the same plane. This is generally true for oligomers with a prime number of subunits (for example, pentamers and heptamers). Thus to find positions of the heavy atoms, we restricted the search to be consistent with the 11-fold symmetry and made a 'molecular replacement' search using the program AMoRe^{28,29} against the isomorphous and anomalous differences with a ring of 11 atoms as a search model. The radius of integration was 20 Å and the radius of the model ring was varied from 6 to 40 Å with stepsize of 0.3 Å. The best correlation for the rotation function was obtained with a model radius of 28.6 Å. The translation search correctly positioned the two rings of the heavy atoms in the unit cell. Details of the procedure will be described elsewhere. Phases were calculated at 3.0 Å resolution using both isomorphous and anomalous differences by the program MLPHARE^{29,30}, with resultant phasing power of 0.8 and figure of merit of 0.229. These phases were improved using the program SQUASH^{29,31}. The Br positions were nearly centric and therefore the anomalous differences could not clearly indicate the correct enantiomorph. It was not possible to follow the chain in the initial map but the solvent boundaries were visible. Two doughnut-shape masks were determined from that map using the program MAMA³² and each subdivided into 11 equal segments. One segment of each mask was used for 11-fold averaging within each oligomer. The initial matrices were derived from Br positions and refined using the initial map with the program MAVER³². Initial correlation coefficients between the segments of each oligomer were in the range 0.3–0.4. The molecular averaging, performed using the program RAVE³², was cycled at 2.9 Å resolution, combining phases from the averaged model with SIRAS phases. The improved phases were used to find more accurate positions for the Br atoms. These were no longer centric and it was possible to choose the correct enantiomorph. New SIRAS phasing at 2.9 Å gave a phasing power of 1.1 and figure of merit of 0.275. The correlation coefficients between the segments improved to 0.40–0.45 and averaging was repeated with improved matrices and map. Combined phases generated a map which allowed us to see the protein backbone in some regions of the density. Partial models built into the map using the FRODO program³³ were used to improve the matrices, and averaging was done in a cyclic manner using $(2|F_o| - |F_c|) \exp(i\alpha_c)$ maps without recombining phases with SIRAS ones. Validity and convergence of the averaging were controlled through the free R -factor³⁴ calculation. Matrix improvement with subsequent averaging was repeated until the matrices were good enough to perform final averaging with phase extension. Improved matrices allowed the use of 22-fold averaging, as the position of the two molecules relative to each other was determined with high accuracy. The 22-fold averaging was cycled with phase extension from 2.9 to 2.2 Å in steps of 0.02 Å using $(2|F_o| - |F_c|) \exp(i\alpha_c)$ maps. The R -factor improved from 50.2 to 25.0. The final map using 2.2 Å phases was of excellent quality and allowed us to build the complete structure, which was refined to a crystallographic R -factor of 0.161 ($R_{\text{free}} = 0.242$) at 2.2 Å resolution using 5-Br-L-tryptophan data. Recently the native data were recollected to 1.8 Å resolution using synchrotron radiation at the X11 beam line, EMBL Hamburg, from a crystal frozen at 110K. The model was refined against these data to a crystallographic R -factor of 0.178 ($R_{\text{free}} = 0.225$). Statistics of the model and of the X-ray data are shown in this table. All residues have allowed main-chain conformational (ϕ , ψ) angles. Seven N-terminal amino acids have poor electron density in each of the 22 independent subunits, indicating their mobility. These residues have been omitted from the current model. The C-terminal residue K75 is visible in the electron density of only 6 subunits, where it is stabilized by intermolecular contacts. Crystallographic coordinates were deposited with the Brookhaven Protein Data Bank¹⁵ under filename 1WAP.PDB.

* The average number of observations of the same reflection.

† The value of the merging R factor between equivalent measurements of the same reflection, $R_I = \sum_i |I_i - \langle I \rangle| / \sum_i I_i$.

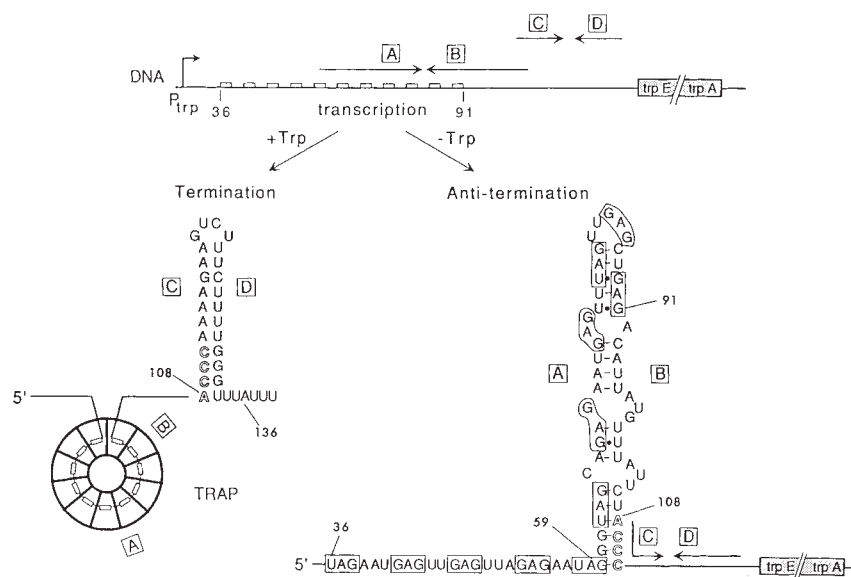
‡ Crystallographic R -factor, $R_{\text{free}} = \sum_o |F_o| - |F_c| / \sum_o |F_o|$. For R_{free} calculation the sum was taken over 1,471 randomly chosen reflections (1% of the total) which were excluded from refinement. All other reflections in the range 10.0 to 1.8 Å were used in the refinement.

§ Correlation between model and $(2|F_o| - |F_c|) \exp(i\alpha_c)$ map.

¶ R.m.s. deviation from the standard values are given with target values in parentheses.

R.m.s. deviation of main-chain and side-chain atoms of the 22 molecules from the average structure.

FIG. 5 Schematic representation of transcription attenuation of the *B. subtilis* *trp* operon. Large boxed letters designate the complementary strands of the terminator and antiterminator. Small rectangular boxes represent the GAG and UAG repeats involved in TRAP binding; these repeats are outlined in the antiterminator structure. Nucleotides 108–111 that overlap between the antiterminator and terminator are shown by outlined letters. Numbers indicate residue positions relative to the start of transcription. Modification of the previously proposed mechanism of attenuation² is shown with the protein represented as a 'wheel' and 11 repeats forming a circle upon binding to surface of the ondecamer.



nucleotide repeats to range from 15 to 52 Å. The potential RNA-binding amino acids are at distances of 18 to 35 Å from the oligomer axis.

Discussion

Ring-like structures with a hole in the middle have been observed in a number of proteins that interact with DNA, for example the dimeric β -subunit of *Escherichia coli* DNA polymerase II (ref. 26) and the amino-terminal fragment of DNA topoisomerase I (ref. 27). It has been suggested these molecules are clamped around a double helix of DNA. The narrowest part of the TRAP hole (Fig. 4c) is defined by the carbonyl oxygens of A66, whose van der Waals surfaces lie at 11.4 Å from the molecular axis. Modelling of B-form DNA into the TRAP hole indicates that the double helix could be threaded through the hole, there being a distance of about 0.3 Å between the van der Waals surfaces of TRAP and the DNA atoms. Another argument for the hypothesis that TRAP wraps around DNA comes from the fact that to stop transcription, TRAP has to find and bind the RNA transcript (nucleotides 36–91) and allow the terminator to form before RNA polymerase passes the terminator region. Thus location of TRAP on the DNA just beside the RNA polymerase would perfectly place a molecule of TRAP close to the RNA transcript being synthesized; however, there is no direct evidence that the molecule of TRAP is wrapped around the DNA.

It is not easy to envisage the evolution of the 11-mer TRAP molecule. As the 11-mer recognizes an 11-fold symmetry structure in the RNA, it would appear to be necessary for the change from a monomeric TRAP to the 11-mer to occur in a single evolutionary step. Given the rotational symmetry axis relating the subunits, intermediate evolutionary stages involving, for example, dimers, trimers or tetramers seem unlikely as there would be nothing to prevent additional subunits binding at the exposed edges of the complex. Perhaps future discovery of homologous proteins with the same or with different oligomer composition will shed light on this.

The binding of eleven U/GAG repeats of the leader region of mRNA transcript suggests that 11-fold symmetry of TRAP oligomer will be retained in the structure of the TRAP/RNA complex. In this model, bound RNA would have a circular structure with a backbone radius of 15–40 Å. We suggest that RNA binds on one of the surfaces of the TRAP doughnut, at regions affected by bound L-tryptophan molecules. Attempts to model the binding of RNA to TRAP are difficult owing to the ill-defined structure of single-stranded RNA. A complete understanding of the interaction between TRAP and *trp* leader RNA and the attenuation mechanism requires crystallographic studies of a complex of TRAP with the mRNA target sequence. Our results provide an essential step towards understanding this mechanism. □

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The optical counterpart of the superluminal source GRO J1655 – 40

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THE accretion of gas from a companion star onto a compact object, such as a neutron star or black hole, can release large amounts of energy. Episodic accretion is generally thought to explain transient X-ray emission, such as that associated with the recently discovered¹ Galactic superluminal source GRO J1655 – 40, and to be connected with the ejection of material at relativistic velocities². The only other known Galactic superluminal source² is difficult to study because it is obscured by intervening gas and dust. Here we report the discovery of the optical counterpart to GRO J1655 – 40, which we estimate to lie at a distance of only 3 kpc. We have identified the precursor on historical photographic plates; at the time of the X-ray burst in August 1994¹, it brightened by 3 mag in the V bandpass. Although the 12-day delay between the X-ray burst¹ and the main radio outburst which marked the beginning of superluminal motion³ poses a puzzle that remains to be explained, our results show that at optical wavelengths the characteristics of GRO J1655 – 40 are similar to those of other accreting-black-hole candidates^{4–7}.

CCD (charge-coupled device) images of 15 fields were taken on 10 August 1994 with the CTIO 0.9-m telescope and a Tek 2,048 × 2,048 CCD microchip. Each image covered 13.5 arcmin on a side: the 15 images together spanned the $0.6^\circ \times 0.6^\circ$ γ -ray error region quoted in ref. 8. The images were compared to digitized images of the UK Schmidt sky survey⁹ taken in 1988. One star was clearly brighter in the CCD images than in the sky survey (Fig. 1); no other star with $V \leq 17$ mag showed such an increase in brightness. Subsequent X-ray observations with the Rosat satellite¹⁰ localized the high-energy source to an 8-arcsec field containing our optical candidate—there is thus no doubt that the optical nova is the same source as the high-energy (X-ray and γ -ray) and radio object described in refs 1 and 3.

On the following night, images of this star were taken in four broadband colours. Calibrating our results with several of Landolt's standard star fields¹¹ yielded magnitudes and colours for the optical nova of $V = 14.40$ mag, $B - V = 1.0$, $V - R = 0.9$ and $R - I = 0.9$. In addition, an image was taken in a 75-Å-wide filter centred at 6,563 Å, and which was therefore sensitive to emission from the hydrogen- α Balmer line. When compared to other stars in the field, the candidate counterpart was found to have a 0.45 mag H α excess with respect to the R magnitude.

On 12 August, we obtained a spectrum of the source with the 1.5-m telescope at La Silla. The exposure time was 55 min, and

the resolution was ~ 10 Å. The slit was aligned with the parallactic angle, and spectra of two spectrophotometric standards¹² were also obtained, so a calibration to absolute flux units could be performed. The spectrum shows a variety of emission lines which have been seen in other X-ray novae in outburst and decline¹³, as well as interstellar absorption features. A simultaneous fit to the Na D-lines (in absorption) and the He I emission line at 5,876 Å gives an equivalent width for the Na D-lines of 4.5 Å. From this, a reddening of $E(B - V) = 1.15$ can be inferred¹⁴. This value is compatible with the equivalent widths (EWs) of the interstellar bands at 4,430 Å (EW, 2.6 Å), 5,780 Å (EW, 1.2 Å) and 6,280 Å (EW, 2.1 Å). A de-reddened spectrum (using a standard extinction law¹⁵) is given in Fig. 2.

The strength of the extinction can be used to estimate the distance to the source¹⁶. Standard relations between absorption-line strengths, interstellar absorption and distance^{17,18} suggest that the distance to the source is compatible with $d \approx 3$ kpc, in agreement with radio observations³. The intrinsic colour of the source ($B - V = -0.1$) is typical of low-mass X-ray binaries in outburst¹⁹, whereas the absolute magnitude ($M_v \approx -2$) is midway between those for outbursts of dynamically confirmed black-hole candidates V404 Cygni ($M_v = -4.5$)¹⁶ and A0620 – 00 and Nova Muscae 1991 (both with $M_v \approx 0$)²⁰. Because the relatively poor spectral resolution makes the deconvolution of the He I emission line from the Na D-line absorption feature difficult, and the signal-to-noise ratio in the 4,430-Å line is low, the magnitudes reported here for GRO J1655 – 40 should be regarded as provisional. In addition, deviations of up to 50% from the relations between line width and absorption, and between absorption and distance, are not unheard of.

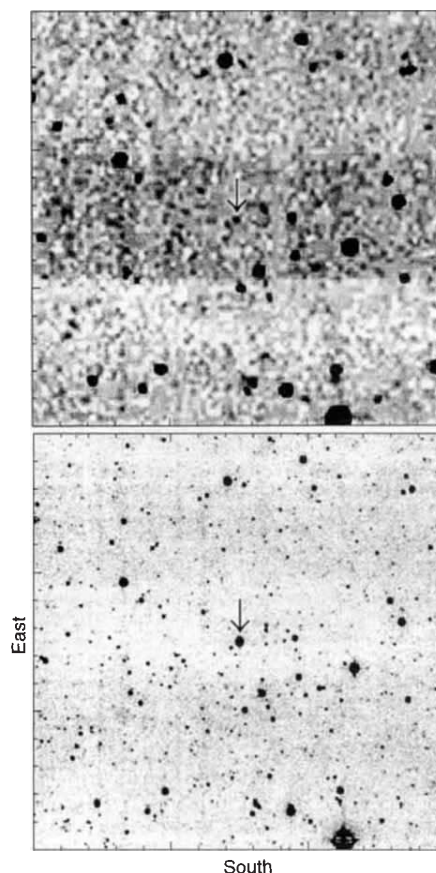


FIG. 1 $3' \times 3'$ area of the sky, from UK Schmidt digital sky survey (top; 4 April 1988), and CTIO observations during the outburst (bottom; 20 August 1994). The images are negatives, so stars are dark spots. Observations are in the V band. The candidate optical counterpart is marked with the arrow.

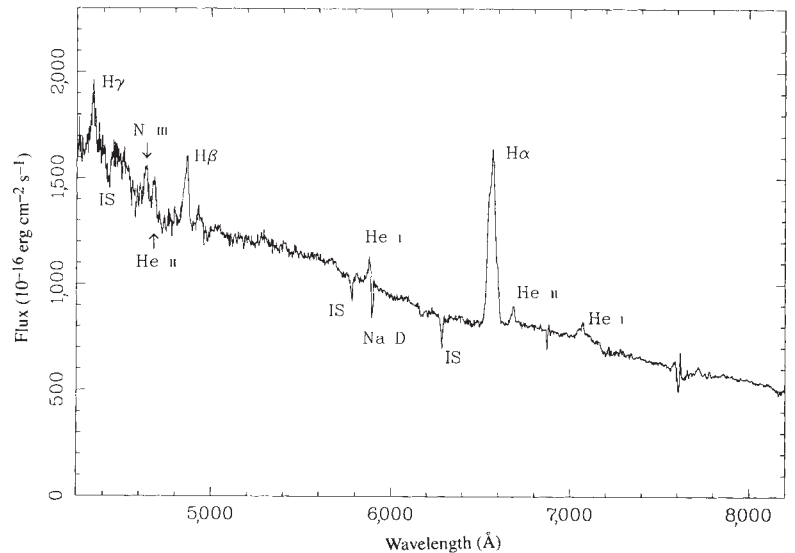


FIG. 2 De-reddened spectrum of the candidate optical counterpart of GRO J1655-40. Prominent emission lines and interstellar absorption features (IS) are indicated.

We monitored the source nightly in the V and I bands until 22 August, and occasionally thereafter until 1 November, after which the object could no longer be observed at night. Our complete lightcurve is shown in Fig. 3. A change in the lightcurve correlated with changes in the radio and high-energy emission¹ was observed on 16–18 August, but no corresponding change in the optical emission was seen in response to the renewed high-energy activity in mid-September. In this case the optical emission may be offset in time from the high-energy emission, just as the radio emission was from the initial outburst³.

During the night of 17 August, a sharp decline and subsequent rise in brightness occurred, accompanied by significant reddening (Fig. 4). The simplest explanation of this behaviour is that it is an eclipse. In response to this event, we monitored the source hourly during the first half of the night (when it could be observed) until the end of our run on 22 August. No similar eclipse-like behaviour was found during this time. If the event on 17 August is interpreted as an eclipse, orbital periods shorter than 1.3 d would have resulted in at least one additional observed eclipse. Longer periods would allow the eclipses to take place when the source was unobservable. The size and duration

of the 17 August event are compatible with a period of a few days. However, in the absence of a second event, the interpretation of Fig. 4 as an eclipse must remain provisional.

We found a precise location of the optical nova using an 8 × 10 inch photographic plate taken on 28 August 1994 at the 26-inch Yale-Columbia refractor at Mt Stromlo observatory. In addition to the nova image, which was ~1 mag above the plate limit, the plate contained images of ten stars from the Position and Proper Motion Catalog²¹ (PPM). The PPM is a list of stars with $V \leq 12$ mag which have locations and proper motions sufficiently accurate to determine their present positions to within ≤ 0.3 arcsec for each individual star^{21, 23}. The PPM stars were used to tie our CCD images into an absolute astrometric reference frame. The position of the optical nova was found to be right ascension 16 h 54 min 00.137 ± 0.015 s, declination -39° 50' 44.90 ± 0.20" (J2000).

The errors quoted include: (1) the formal errors based on the quadrature sum of the uncertainty of the CCD nova position in the Mt Stromlo plate system and the uncertainty of the mean transformation to PPM celestial coordinates; (2) possible zonal offsets between the PPM catalogue and the fundamental

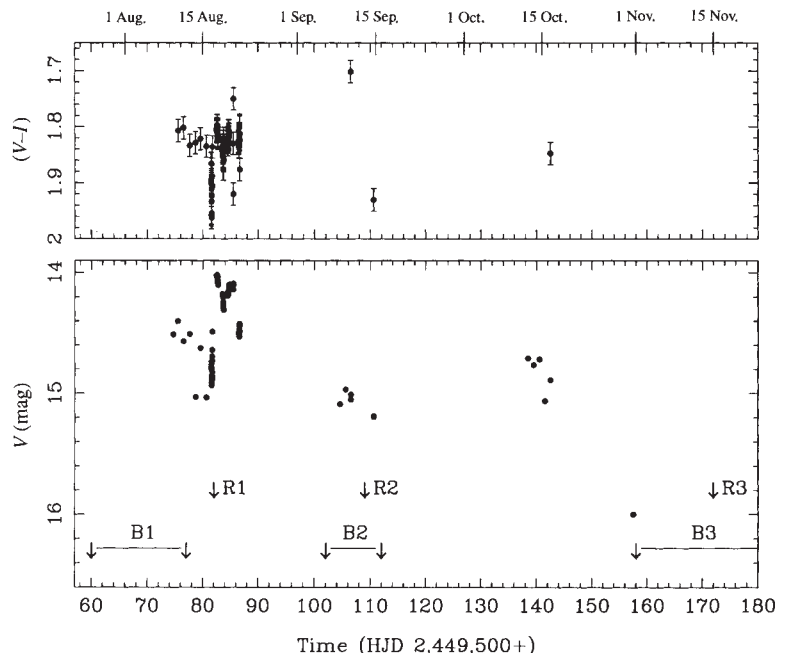


FIG. 3 V-band lightcurve and V-I colours. Random statistical errors are very small in all cases, but systematic errors of ~0.02 mag are expected from the profile fitting. Times are given in heliocentric julian days (HJD) and calendar dates of 1994. The times of the peaks in the radio flares are labelled R1 to R3. The beginnings and endings of the major γ -ray flares reported by the BATSE team are labelled B1 to B3 (B3 ended shortly after day 2,449,685). The increase in brightness between 16 and 18 August corresponds to the peak of the first large radio event, but there is no corresponding optical response to renewed activity in mid-September. Note the sharp decline in the last two weeks of October, shortly before the third BATSE event.

system²²; and (3) an estimate of the maximum size of an undetected bias with magnitude for the plate measurements. The position is within the 0.8-arcsec uncertainty of the stationary component of the radio source²⁴.

We have also analysed a pre-outburst plate of the region taken in 1968 with the 50-cm double astrograph at El Leoncito, Argentina, as part of the Yale/San Juan southern proper-motion survey. The precursor is clearly visible at $V=17.3$ mag, which is 1 mag above the plate limit. The position of the precursor is 0.35 ± 0.20 arcsec away from the position of the optical nova. This offset could easily be due to errors in the PPM proper motions over the 26-year plate epoch difference. A true proper motion of the source of a few tenths of an arc second might also be expected. There are only ~ 800 stars of $V \leq 18$ mag in the $180' \times 180'$ CCD image, so the *a priori* probability that an unrelated star would be located within a few tenths of an arc second of the radio position of GRO J1655–40 is small. Therefore we conclude that the precursor candidate is likely to be associated with the nova.

When compared to previous transient high-energy emissions (generally referred to as 'transients') from sources containing dynamically confirmed black-hole candidates, GRO J1655–40 shows many similarities, and some significant differences. The combination of radiation at energies >100 keV, together with the optical spectrum, luminosity and de-reddened colour of GRO J1655–40, has been observed in A0620–00, Nova Musc 1991, V404 Cyg and Nova Ophiuchi 1977, all of which have been shown to contain compact objects with mass $M \geq 3M_{\odot}$ (refs 4–7). No transient with these characteristics has exhibited short X-ray bursts or regular pulsations, phenomena which are generally associated with neutron stars. (Sources such as Centaurus X-4, which have been found to emit X-ray bursts, have not been observed to emit γ -rays). On the other hand, superluminal radio jets and highly variable high-energy lightcurves like that described in ref. 1, have not been seen in previous dynamically confirmed black-hole candidates. Furthermore, the quiescent optical counterpart of GRO J1655–40 is much brighter than

those of the above-mentioned sources. Thus although the characteristics of the optical outburst of GRO J1655–40 strongly resemble those of previous black-hole candidates, other aspects of this source seem to be unique. $\square$

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Correlations between X-ray outbursts and relativistic ejections in the X-ray transient GRO J1655–40

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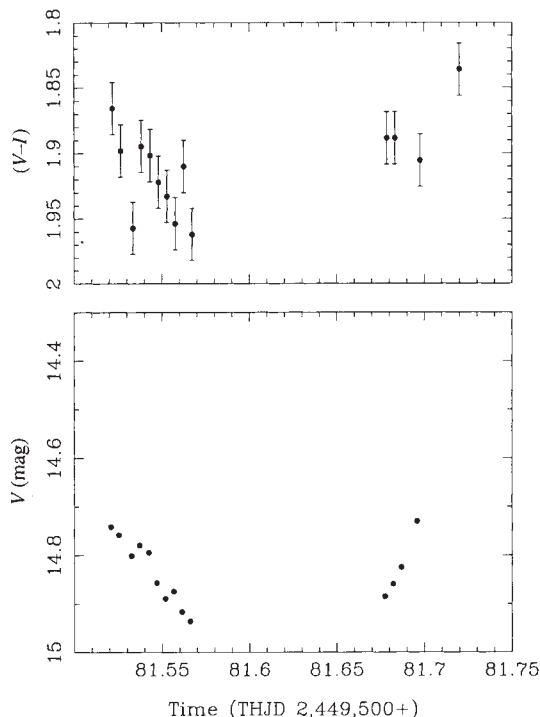


FIG. 4 V–I colours (top) and V-band lightcurve (bottom) for the night of 17 August. Note the eclipse-like behaviour, which was discovered in subsequent analysis. Unfortunately no data are available during the minimum, and no recurrence was detected.

ALTHOUGH objects that emit radio jets have been known for many years, the physical mechanism responsible for the jets has been unclear. Accretion of mass onto a compact object (such as a black hole) has often been invoked in models of their formation¹. X-ray emission from such sources is a potentially powerful probe of the processes taking place, because it seems to arise much closer to the central object. Here we report the detection of X-ray and radio emission from the recently discovered^{2,3} transient source X-ray Nova Scorpii 1994 (GRO J1655–40). The radio outbursts, presumably reflecting the feeding of material into relativistic jets, generally follow bursts of hard X-ray emission (20–400 keV) with

a delay that varies from a few days to about two weeks. This suggests that the mechanism behind the X-ray emission is not related to the ejection process in a simple way. Nevertheless, GRO J1655–40 may be the best example of a compact object/accretion-disk system in which models of jet formation and X-ray production can be tested directly.

GRO J1655–40 was first detected on 27 July 1994 with the Burst and Transient Source Experiment (BATSE)⁴ on board the Compton Gamma Ray Observatory (CGRO). The transient source was clearly distinguishable from nearby hard X-ray sources such as OAO 1657–415². The source was located initially to 0.3° uncertainty using Earth occultation imaging⁵. Using additional BATSE data, the uncertainty was reduced³ to $\sim 0.1^\circ$, in agreement with the reported optical location⁶.

Figure 1a shows the GRO J1655–40 X-ray lightcurve in the 20–100 keV energy band obtained using the Earth occultation technique. The X-ray lightcurve is dominated by three main outbursts, the first between 28 July and 15 August, the second between 6 and 16 September, and the third which was also the most intense, between 1 November and 25 December. (The third episode could also be considered as two separate, closely spaced outbursts.) The rise in hard X-rays of GRO J1655–40 largely occurred in less than one day, between 27.75 and 28.25 July UT, although there was no well defined maximum during the first outburst. Similarly, the second outburst showed a fast rise on 6 September. There is some evidence for a low-level flux preceding the first and second outbursts, but its significance is marginal, given the combined statistical and systematic error determined for the daily averages in Fig. 1a. The source showed no emission lasting more than 5 d above the level of $0.03 \text{ photons cm}^{-2} \text{ s}^{-1}$ in the 20–100 keV band ($\sim 10\%$ of the peak flux) for the 50-day interval before 27 July.

Figure 1b shows the total radio flux at 1.49 GHz measured with the National Radio Astronomy Observatory Very Large Array (VLA). A flux increase in the 1.49 GHz band is associated with each X-ray outburst, but the time of maximum radio flux varies greatly among outbursts: the radio peak for the first outburst occurs about 10 days to 2 weeks after the peak X-ray flux in early August, but in later outbursts, the peak radio flux

appears to follow the X-ray maxima by only 1–2 d. The latter part of the third X-ray outburst in late November and early December, which shows very intense X-ray fluxes, is reflected only as a modest slowing in the decline of the radio flux.

Times when major radio ejection events began are also shown in Fig. 1. These times precede the maximum radio flux by ~ 1 week and correspond reasonably well to episodes of enhanced X-ray flux, but, as can be seen for the first and third outbursts, not with the peak X-ray flux determined from the daily averages.

To examine these putative relationships in more detail, we have plotted the X-ray and radio data during the first and second X-ray outbursts in Fig. 2 on an expanded scale to illustrate the short-term flux variability of GRO J1655–40. In early August (Fig. 2a), the X-ray source began an overall decline with superimposed shorter timescale variations (hours). Coincident with the X-ray decline, the Australian Molonglo Radio Observatory reported⁷ a rise in flux at 843 MHz from 0.9 to 7.1 Jy. Observations with the VLA, which began on 12 August, also showed an increase in the 1.49, 4.9 and 14.9 GHz bands as shown in Fig. 2a. The radio spectrum, which first exhibited an optically thick, relatively flat spectrum on 12 August, evolved to an optically thin synchrotron spectrum by 18 August.

The second outburst (Fig. 2b) had a slightly more complicated, but similar, behaviour. The 1.49 GHz and 843 MHz radio fluxes showed increases at the times of prominent X-ray dips on 11 and 13–14 September, respectively. Although the radio coverage is less complete than that of the first outburst, the data indicate that the radio spectrum steepens between 11 and 13 September, and possibly continues to steepen until 14 September. Combined Molonglo Observatory and AT Compact Array observations also support a steepening of the radio spectrum⁸. The optically thick spectrum observed by the VLA on 11 September appears to be associated with a new component in the central radio source that is distinct from the extended components seen in the VLA images from the initial outburst⁹.

To search for possible periodic behaviour associated with the short-term X-ray variability, such as a possible binary orbital signature or correlations with the kinematic model being developed for the radio jets⁹, occultation fluxes at 0.1-d intervals were

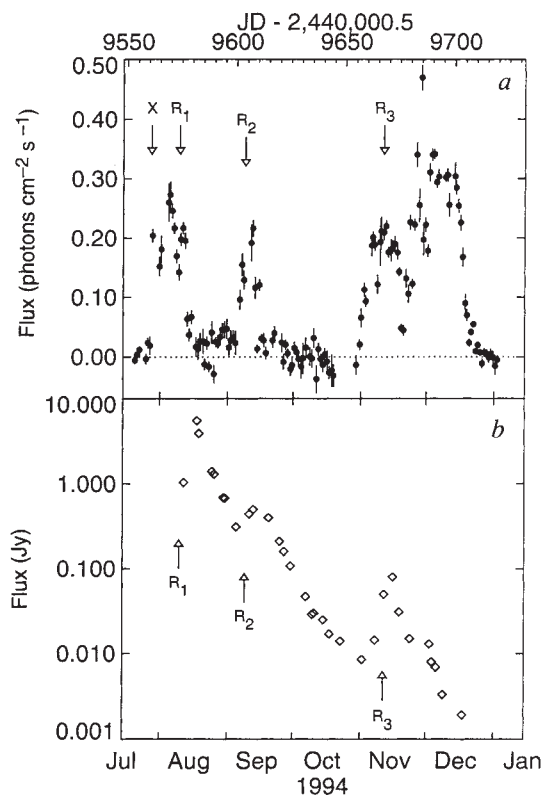


FIG. 1 a, BATSE lightcurve for GRO J1655–40 in the 20–100 keV energy band. Individual occultation measurements are used to compute the daily average fluxes shown. To obtain the photon flux, source count rates are extracted in each energy channel from occultations of the source during a single day (typically ~ 20 occultations are usable); then, the averaged rates are fit to a power law convolved through the response functions of the BATSE detectors. Other bright sources in the region are accounted for by simultaneously fitting their occultation step features. The indicated errors are statistical; the systematic error is estimated to be 5% of maximum intensity of the first outburst ($\sim 0.3 \text{ photons cm}^{-2} \text{ s}^{-1}$, 20–100 keV) due to variability of nearby sources. “X”, “R₁”, “R₂” and “R₃” indicate, respectively, times of the initial rise of the X-ray flux, and the onset of major plasma ejection episodes (jet formation) as determined from VLBA images⁹, where R₁ = (truncated Julian date, TJD) $9,574 \pm 1$ (10 August 1994), R₂ = $9,604 \pm 2$ (9 September 1994) and R₃ = $9,668 \pm 5$ (12 November 1994). The jets appear as a semi-continuous stream of radio-emitting plasma with brighter portions which display clear proper motion⁹ corresponding to relativistic velocities at the estimated distance¹² of 3.5 kpc. Some features within the jets can be tracked in separate observations for several days. The times are obtained by extrapolation of the proper motion of the radio-emitting plasma back to the central source⁹. b, 1.49 GHz radio lightcurve for GRO J1655–40 measured with the VLA. The fluxes represent the total intensity of the extended radio source, and are plotted logarithmically to enhance the visibility of secondary features in the lightcurve.

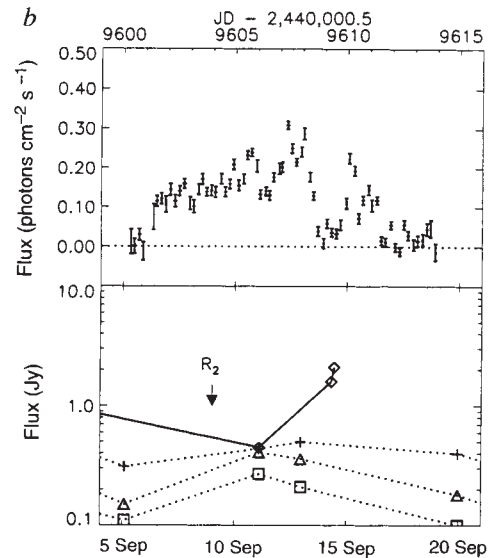
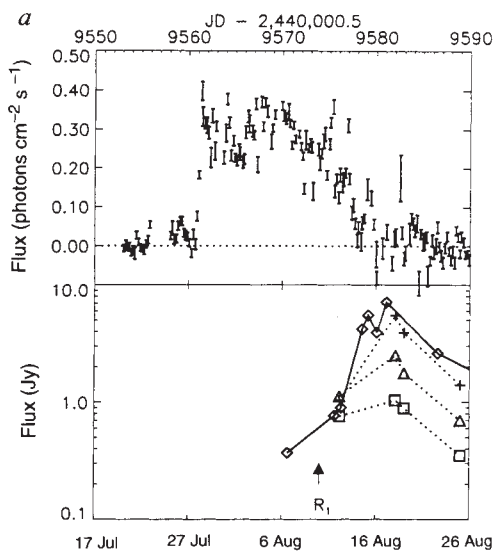


FIG. 2 Lightcurves for GRO J1655–40 in the 20–430 keV band for the first (a) and second (b) outbursts. BATSE occultation data are shown for 0.2-d (4.8-h) sampling intervals which typically include ~ 2 –6 flux measurements. To achieve maximum statistical accuracy, the flux has been calculated from a power-law fit assuming a fixed spectral index of -3.1 and a variable normalization. Simultaneous radio measure-

ments are also shown from the Molonglo observatory at 843 MHz (diamonds) and the VLA at 1.49 (crosses), 4.9 (triangles) and 14.9 GHz (squares). The 843 MHz flux on 11 September (0.45 Jy) is an upper limit based on the optically thick spectrum which peaked^{8,9} at 5 GHz. As described in Fig. 1, R_1 and R_2 indicate the onset of major plasma ejection episodes lasting several days.

processed with a Lomb algorithm for unevenly sampled data¹⁰ to obtain a histogram of periods. This revealed no significant periods between 0.5 and 50 d.

BATSE spectra are shown in Fig. 3 for two time intervals during the first and second outbursts of GRO J1655–40. The spectrum was generally well fitted by a single power law whose photon spectral index showed some evolution during each outburst. The index reached a minimum value of ~ -3.1 , but ranged as high as -2.5 , generally when the intensity was lower; however, no strict hardness–intensity relationship was observed. The spectrum for 4–9 August corresponds to the X-ray peak of the first outburst, which shows no evidence for a cutoff below 400 keV. The Oriented Scintillation Spectrometer Experiment on the CGRO reported¹¹ significant emission between 4 and 9 August up to 600 keV.

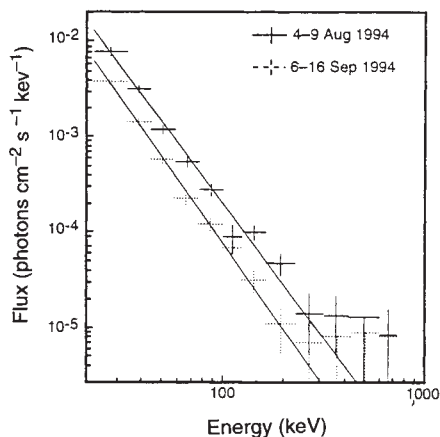


FIG. 3 Energy spectra for GRO J1655–40 from 4 to 9 August (first outburst) and 6 to 16 September (second outburst) 1994. The power-law spectral indices for the fits shown were -2.8 ± 0.06 and -3.0 ± 0.1 for 4–9 August and 6–16 September, respectively. An extrapolation of the spectrum of the first outburst down to 1 keV for a 3.5 kpc source distance¹² yields a luminosity at or exceeding the Eddington luminosity for a $1 M_{\odot}$ black hole.

Based on the estimated¹² distance of 3.5 kpc (lower and upper limits are 3 and 5 kpc, respectively¹³), we derive a 20–100 keV peak luminosity from Fig. 3 of 1.6×10^{37} erg s⁻¹.

GRO J1655–40 seems to be one of a class of objects known as ultra-soft or soft X-ray transients. The more popular, but less accurate, name is X-ray novae. These objects have been shown to be binary systems with a low-mass, late-type main-sequence star and a compact object surrounded by an accretion disk¹⁴. If the companion star is bright enough, Doppler shifts in the visible spectrum signifying the orbital motion of the binary can be observed once the accretion disk returns to its quiescent state. The measured shifts lead to an optical mass function which can be used to set a lower limit on the compact object mass. Three systems have been firmly established to contain a compact object of $3 M_{\odot}$ or larger, and thus are believed to contain black holes¹⁴.

The classification of GRO J1655–40 as an X-ray nova is supported by a variety of observations, mainly the fast rise of the hard X-ray flux, the gradual decline of the radio flux (characteristic of an adiabatically expanding synchrotron radio source), and the appearance of an optical nova with a characteristic low-mass X-ray binary spectrum⁶. Also, multiple secondary radio, optical and X-ray outbursts are frequently seen in such objects¹⁵. However, some of its X-ray and optical properties identify this source as a peculiar X-ray nova, irrespective of its radio properties. Usually secondary outbursts in X-ray novae tend to appear as perturbations on the decline of the main outburst, which is often exponential in shape. This is in stark contrast to the X-ray lightcurve shown in Fig. 1. The early outburst spectrum, as shown in Fig. 3, is also somewhat steeper than the usual spectra of X-ray novae. The optical counterpart of GRO J1655–40 (ref. 6) shows a change in magnitude from quiescence to outburst of $\Delta V \approx 3$ mag, peaking at a visual magnitude of 14.4. More typically¹⁵, $\Delta V \approx 6$ –7. Nevertheless, the latter suggests that it will be possible to obtain a mass estimate for the compact object when this system returns to quiescence.

The identification of the compact object in GRO J1655–40 as a neutron star or black hole depends on further studies. In lieu of an optical mass function, we can study the source's high-energy spectral and temporal properties. The detection of pulsations or X-ray bursts would immediately indicate the presence of a neutron star; however, it is not often that these are found

in X-ray novae. Beyond this, there are properties that, taken together, are usually considered to be indicative of black-hole systems¹⁴. They are: (1) an ultra-soft spectrum in the 1–10 keV band, (2) a hard spectral component which extends to several hundred keV, and (3) broad-band, short timescale variability ($1\text{--}10^{-3}$ s), usually when the hard spectral component is present. The TTM–Kvant–Mir team reported an ultra-soft spectrum for GRO J1655–40 on 24 and 25 September with a black-body temperature¹⁶ of 1 keV. Rosat observations¹⁷ show flux variability on timescales down to 0.1 s in the 0.1–1 keV energy band. Although more detailed studies of possible short-timescale variability of the source are underway (D. Crary *et al.*, manuscript in preparation), the standard all-sky monitoring routines performed daily on BATSE background data showed no prominent variability or pulsed emission from the direction of GRO J1655–40 in the time range of 3–300 s at 10% of the peak flux level observed in Fig. 1. The current observational constraints therefore favour the presence of a black hole in GRO J1655–40.

Theoretical arguments indicate that jet-like or collimated supersonic flows can arise from radiation-pressure dominated disks, where mass flow to the inner disk can feed loosely collimated jets of material¹.

A consistent relationship between the hard X-ray and radio fluxes and the plasma ejection times is observed in three distinct episodes of GRO J1655–40. This supports the interpretation that feeding of material into jets occurs in the inner disk, as that is also the most likely site of hard X-ray production. The varying delay times between the X-ray and radio secondary peaks, and the general tendency away from a simple one-to-one relationship between fluxes in the two wavelength bands, indicate that the ejection mechanism is not strictly determined by a critical luminosity as determined from the hard X-ray flux alone. We note that intense soft X-ray fluxes have been detected¹⁶ when the hard X-ray flux as determined by BATSE was relatively weak. Further, we find that the hard X-ray flux shows significant variability on a timescale of hours, and a degree of anti-correlated behaviour with the radio flux, usually after the X-ray flux has reached a maximum. This would suggest that the emission of X-rays and γ -rays is being suppressed by a change of state in the inner disk rather than simply decreasing in response to a reduction in accretion rate.

It is interesting to compare this source with the transient source GRS 1915+105. This object was discovered in August 1992 with GRANAT/WATCH¹⁸ and reported recently by Mirabel and Rodríguez¹⁹ to be a radio source with superluminal jets. Although GRS 1915+105 has a hard X-ray spectrum²⁰ similar to GRO J1655–40, it has not been considered an X-ray nova due to its long risetime²¹. It has undergone long-duration flares since 1992 in hard X-rays²¹ and radio flares up to 1 Jy from 1992 to 1994¹⁹. It is variable over hours to days, and episodes of strong X-ray emission lasting several weeks are interspersed with quiescent periods (K. Deal *et al.*, manuscript in preparation). The dramatic variability of the hard X-rays seen in these two transients would appear to be related to the presence of the radio jets; however, more detailed correlative studies will have to be done. □

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Waves from the collisions of comet Shoemaker–Levy 9 with Jupiter

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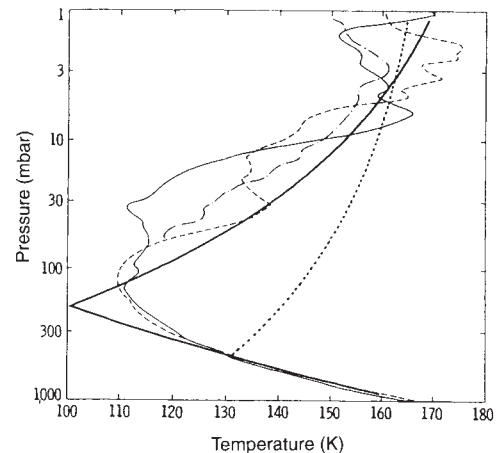
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OBSERVATIONS of the collisions of the fragments of comet Shoemaker–Levy 9 with Jupiter provided an unprecedented opportunity to probe the depths of the planet's atmosphere. Images taken by the Hubble Space Telescope revealed circular rings surrounding five of the impact sites¹. The rings were observed for up to 2.5 hours after the impacts and spread at a constant velocity of 450 m s^{-1} . There are three types of disturbance that might explain these observations: acoustic waves trapped at the tropopause temperature minimum², gravity waves propagating vertically and horizontally in the stratosphere³, and gravity waves trapped in a stable layer which acts as a horizontal waveguide and is located within the hypothesized tropospheric water cloud⁴. Here we show that only the last of these phenomena can match the speed and relative amplitude of the observed waves, with the requirement that the impacts were deep and the stability of the trapping layer is large. The origin of the stable layer is still uncertain, but if it is produced by moist convection in the water cloud, then the ratio of oxygen to hydrogen on Jupiter must be surprisingly large—approximately ten times that on the Sun.

Rings were seen after impacts A, E, G, Q1 and R. In a plot of ring radius r versus time t , the points from the different impacts all lie on the same straight line. The slope gives the speed, which is 450 m s^{-1} . The intercept gives the initial radius, $r = 500\text{ km}$ at $t = 0$. Impact sites E and G also have a faint inner ring, the speed of which is uncertain but is in the range $180\text{--}350\text{ m s}^{-1}$. The ring material seems to be impact debris. Images taken through a methane filter, in which high-altitude clouds appear bright, suggest that the ring material is located in the stratosphere somewhere between the 1- and 300-mbar levels^{1,5}.

The observed constant velocity suggests that the rings are propagating waves. The velocity would decrease with time if the particles were carried (advected) with the disturbance. This is because advection is a nonlinear process—the speed of propagation depends on the amplitude. Because the amplitude decays with time due to geometrical spreading, the speed should decrease with time if advection occurred. West *et al.*⁵ argue convincingly that the particles are created by condensation and destroyed by evaporation as the wave moves past. Other evidence of wave-like behaviour is that the points from different impacts fall on the same straight line in the plot of radius versus time. If material were flowing outwards behind a shock wave or other nonlinear disturbance, the speed would depend on the

FIG. 1 Temperature profiles as measured by Voyager (dashed, dot-dashed, and light solid lines)⁷ and as used in the gravity-wave model (dotted and heavy solid lines). The wiggles in the measured profiles are real, making it difficult to define the 'true' profile. The model profile⁴ on the right (dotted line) gives the right amount of leakage of wave energy into the stratosphere⁹. The profile on the left (heavy solid line) provides a better fit to the temperatures in terms of least-squares error. Our conclusions are the same regardless of which profile is used.



energy of impact, and the points would fall on different lines. For a linear wave, the speed depends only on properties of the medium. Nonlinear effects near the source could account for the finite initial radius of the ring.

We have calculated the amplitudes and speeds of sound waves and gravity waves for an impact energy of 10^{27} erg. We use linear theory, so amplitude scales with the energy. By "amplitude" we mean the lagrangian temperature perturbation at the 50-mbar level, near where the impact debris resides. Sound-wave amplitudes must be small, because no waves were seen travelling at the speed of sound. Even at the tropopause, where the temperature has its minimum value of 110 K, the speed of sound is 770 m s^{-1} , which is too fast to match the observations.

Following Kanamori², we represent sound waves from the impacts as a superposition of vertical normal modes. The fundamental ($n=0$) mode is incompressible, and has no temperature perturbation⁶. The $n=1$ mode represents energy trapped in the horizontal waveguide located at the temperature minimum near the 100-mbar level (that is, where $P=100$ mbar). The temperature profile is a digitized representation of the Voyager data⁷ shown in Fig. 1. The largest amplitude is associated with the trapped mode, and is $\pm 35 \text{ K}$ for a source near the temperature minimum (Fig. 2). For deeper sources the response is smaller, for example, $\pm 0.65 \text{ K}$ and $\pm 0.20 \text{ K}$ for sources at the 5- and 10-bar levels, respectively. As sound waves were not seen, the energy release must have been deep. A deep source is not inconsistent with the appearance of the fireball⁸ at later times and higher altitudes.

Following Ingersoll *et al.*⁴, we study gravity waves for a hydrostatic fluid on a rotating plane—an f -plane, in which the interaction of rotation and spherical geometry is neglected. Vertical structure follows Voyager data⁷ in the stratosphere and a moist

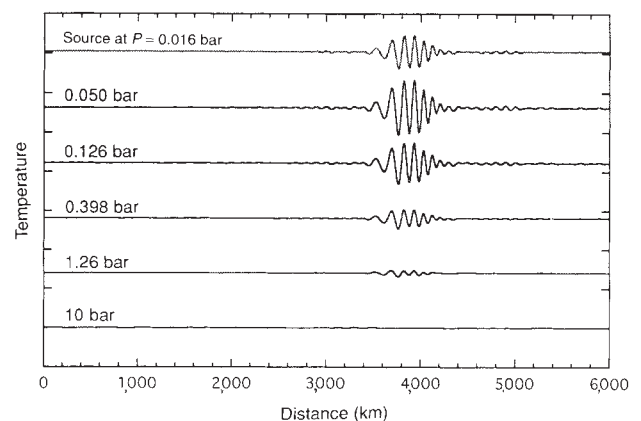
adiabat in the troposphere⁹, both fitted to simple analytical functions (Fig. 1, and Fig. 1 of ref. 4). The top of the model is an isothermal stratosphere extending to infinity at temperature $T_\infty = 185 \text{ K}$. The response to an impact includes both stratospheric gravity waves (SGWs), which propagate vertically, and tropospheric gravity waves (TGWs) which are trapped in the water cloud. The latter has a stable (sub-adiabatic) structure that is maintained by latent heat release and therefore depends on the jovian water abundance.

We define $e_{\text{H}_2\text{O}}$ as the enhancement factor of water relative to the solar abundance, that is, the jovian O/H ratio relative to that on the Sun. The speed of the TGW varies⁴ as $e_{\text{H}_2\text{O}}^{1/2}$, and for $e_{\text{H}_2\text{O}}=1$ the speed is 130 m s^{-1} . So if the rings were associated with TGWs, the observed speed of 450 m s^{-1} would imply $e_{\text{H}_2\text{O}} \approx 10$.

Figure 3 shows three of the heating profiles used in our calculations⁴. The abscissa is J , the energy deposited per unit pressure interval, and is related to energy/mass. All three profiles have $\int J dP = 10^{27} \text{ erg}$. (We use $\alpha^{-1} = P_m$, which is defined following equation (3) of ref. 4.) The cloud base is deep, near the 20-bar level, because we have assumed $e_{\text{H}_2\text{O}} \approx 10$. The $\alpha=0$ profile has energy deposited near the cloud base, the $\alpha=1$ profile is an intermediate case, and the $\alpha=5$ profile has energy deposited mostly in the stratosphere. In all cases heating is zero below cloud base.

Figure 4 shows our calculation of gravity waves 2 h (7,200 s) after the impact, using the temperature profile shown as a heavy solid line in Fig. 1 with $e_{\text{H}_2\text{O}} \approx 10$. The abscissa is radial distance r from the impact point. The ordinate is the lagrangian temperature perturbation at the 45-mbar level. The pulse at $r = 3,300 \text{ km}$ is the stratospheric 'tail' of the TGW travelling at 450 m s^{-1} . As in Fig. 2 of ref. 4, the disturbance amplitude is considerably

FIG. 2 Temperature perturbation versus distance for the acoustic-wave model, 5,400 s (1.5 h) after the impact; the energy of impact is 10^{27} erg. The curves show the temperature change experienced by a parcel at the 126-mbar level, for six different altitudes of energy input (all the energy goes in at one level); on the vertical axis, one division represents 50 K. The response drops as the depth increases. For a source at 5 bar the amplitude is $\pm 0.65 \text{ K}$; for a source at 10 bar the amplitude is $\pm 0.20 \text{ K}$. The speed of the wave packets is 725 m s^{-1} , which is faster than that seen in the Hubble Space Telescope images.



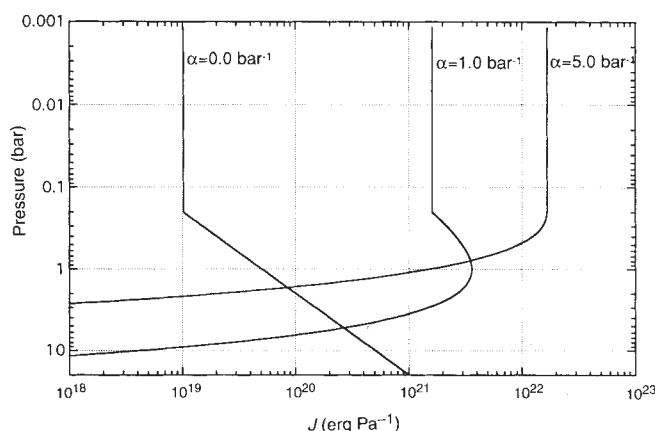


FIG. 3 Heating profiles for the gravity-wave model, defined following equation (3) of ref. 4, with $\alpha = 1/P_m$. Heating is expressed in terms of J , the energy deposited per unit pressure interval, with $\int J dP = 10^{27}$ erg. The model has 10 times the solar abundance of oxygen. The base of the water cloud is at 20 bar, and $J = 0$ below this level.

larger at 45 mbar than it is at 1 bar or at the ammonia cloud tops near 500 mbar.

The disturbance at $r = 6,700$ km is the leading edge of the SGW. Its speed is 930 m s^{-1} . We have run simulations where $e_{\text{H}_2\text{O}}$ has ranged from 0.5 to 10; we have tried putting all the heat at one level, varying the pressure of the level from 10 mbar to 10 bar; we have increased the pressure at the tropopause by a factor of three (Fig. 1, dotted line); and we have varied T_∞ . We find that the speed of the TGW depends only on $e_{\text{H}_2\text{O}}$. The speed of the SGW depends only on T_∞ , varying as $T_\infty^{1/2}$, and does not match the 450 m s^{-1} observed speed for any reasonable profile. Also in all our simulations, the amplitude of the SGW is less than that of the TGW. We conclude that the observed waves are TGWs, not SGWs.

The numerical model of Harrington *et al.*³ has five vertical layers. The top layer, with its base at 16 mbar, is a 'sponge' whose dynamics are dominated by Rayleigh friction. In contrast to our results, Harrington *et al.*³ found SGWs with speeds of 400 m s^{-1} . We can reproduce their result by replacing our semi-infinite stratosphere with a free surface (pressure perturbation = 0) at 16 mbar. The free surface reflects upward-propagating

waves; a sponge does the same, in models with low vertical resolution¹⁰. Reflection creates a stratospheric waveguide that changes the wave speeds. But in the real atmosphere, fast waves like these are not reflected.

In our model there is a second wave that travels at one-third the speed of the first (Fig. 4). These are⁴ the second and first modes of the tropospheric waveguide, spanning three quarter-cycles and one quarter-cycle, respectively, from cloud base to tropopause. The two modes might be related to the inner and outer rings, although the ratio of the two speeds (1:3 in the model) does not exactly match the observed ratio ($\sim 1:2$). This could be a failure to match the shape of the tropospheric temperature profile. The problem goes beyond our use of simple analytical functions, as the true profile is unlikely to follow a moist adiabat exactly.

Other inferences about the abundance of jovian water tend to support $e_{\text{H}_2\text{O}} \geq 1$, but there are major uncertainties. Bjoraker *et al.* observed water spectroscopically and inferred $e_{\text{H}_2\text{O}} \approx 1/50$. Analysing the same data, Carlson *et al.*¹² conclude that $e_{\text{H}_2\text{O}} \geq 1$, and argue that $e_{\text{H}_2\text{O}} = 10$ fits the data equally well (see Fig. 9 in ref. 12). Voyager and Earth-based observations¹³ give $e_{\text{CH}_4} \approx e_{\text{NH}_3} \approx 2$ and $e_{\text{He}} \approx 0.7$. Analysis of the gravitational harmonics of Jupiter¹⁴ suggests at least a five-fold enhancement of relatively heavy elements like oxygen, carbon and nitrogen. This predicted enhancement drops by a factor of two if Jupiter has a sub-adiabatic (radiative) zone in the 1–40 kbar pressure range, as suggested by Guillot *et al.*¹⁵.

Our work will be tested when the Galileo spacecraft encounters Jupiter in December 1995. We predict that it will find a deep stable layer extending down to the 20-bar level. Our best guess is that this layer is maintained by moist convection, with $e_{\text{H}_2\text{O}} \approx 10$. Such a result has important implications for our understanding of the origin of Jupiter, its internal history, and the chemistry and meteorology of its current atmosphere. □

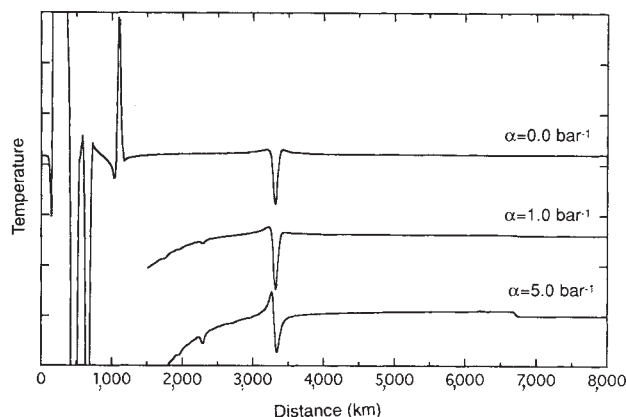


FIG. 4 Temperature perturbation at the 45-mbar level for the gravity-wave model, 7,200 s (2 h) after the impact; the energy of impact is 10^{27} erg. The unit is 1 K per division on the vertical axis. The abscissa is radial distance r from the impact point. The pulse at $r = 3,300$ km is the tropospheric gravity wave (TGW) that propagates in the water cloud. The water abundance ($e_{\text{H}_2\text{O}} \approx 10$) was chosen so that the speed of the TGW matches the 450 m s^{-1} observed speed. The pulse at $r = 1,100$ km is the second mode of the TGW. Its amplitude is off-scale for the lower two curves. The disturbance at $r = 6,700$ km is the stratospheric gravity wave (SGW). Its amplitude increases with α as the altitude of energy deposition rises (Fig. 3), but is always less than that of the TGW. The speed of the SGW is 930 m s^{-1} , which is more than twice the observed speed.

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Near-surface alignment of polymers in rubbed films

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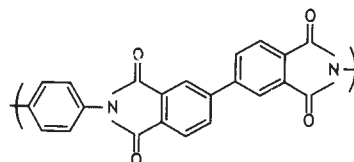
RUBBED polymer films (generally polyimides) are used in flat-panel displays to control the alignment of liquid crystals in contact with the polymer^{1–8}, a phenomenon first discovered by Mauguin¹ in 1911. Buffing the film with a cloth produces liquid-crystal alignment in the rubbing direction. Several mechanisms have been proposed to explain this effect. The generation of microgrooves or scratches on the polymer surface during rubbing has led to the suggestion that alignment is the result of long-range elastic effects induced by these surface features^{3–5}. Others have suggested that the polymer chains near the surface are aligned during rubbing and that these then serve as templates for liquid-crystal alignment^{6–13}. Other studies^{10–12} have implied that both mechanisms might be operative. Here we present X-ray scattering measurements which show unambiguously that rubbing a polyimide film causes near-surface alignment of the polymer molecules. For a film 200 nm thick, most of the polymer chains within a thin surface region (about 5 nm thick) are aligned in the rubbing direction; for a 6-nm film essentially all of the chains are aligned within 20° of the rubbing direction. This marked orientation of the near-surface chains at temperatures far below the bulk glass transition temperature shows that the mechanical properties of the near-surface region differ significantly from those of the bulk polymer.

Previous studies^{6–13} have used bulk-sensitive methods such as optical birefringence and infrared dichroism to infer that rubbing polymer films causes molecular alignment. From measurements as a function of film thickness, it has been suggested that this alignment occurs near the polymer surface^{6–8}. Such an interpretation, however, ignores the possibility that the extent of orientation of the polymer depends on film thickness (an effect that our data does in fact show).

To obtain direct information on the effect of rubbing on the near-surface of polyimide films, we have used grazing-incidence X-ray scattering (GIXS)^{14–16}. This technique exploits the fact that the index of refraction for X-rays is slightly less than unity; this means that there exists a critical angle, α_c , below which the X-rays are totally externally reflected. Figure 1 illustrates the GIXS geometry. X-rays are incident upon the polymer surface at a grazing angle, α . For $\alpha < \alpha_c$, the X-ray wavefield in the film is evanescent and is limited to the top ~5 nm of the polymer film. The penetration depth of the evanescent field and the projection of the incident beam onto the film surface define a scattering volume that is limited to the near-surface region of the polymer. Conversely, for $\alpha > \alpha_c$, the X-rays penetrate the film to a depth that is limited primarily by their absorption in the material. Therefore, scattering measurements above and below α_c provide a means of distinguishing between the bulk and the near-surface structure of the polymer. In scattering experiments, the intensity of the scattered radiation is determined by structural correlations parallel to the scattering vector $\mathbf{Q}$ which is the difference between the scattered and incident X-ray wavevectors, $\mathbf{k}'$ and $\mathbf{k}$ (see Fig. 1). The magnitude of $\mathbf{Q}$ is given by $(4\pi/\lambda) \sin \theta$ where λ is the X-ray wavelength (here 0.1703 nm) and 2θ is the angle between the incident and scattered X-rays. Conse-

quently, measurements of the scattered intensity with $\mathbf{Q}$ parallel or perpendicular to the rubbing direction permit a determination of the structure parallel or perpendicular to this direction, respectively.

The polyimide used in this study was derived from the polyamic acid ester of bisphenylenedianhydride and *p*-phenylenediamine, denoted BPDA-PDA, and has a quoted¹⁷ glass transition temperature, T_g , in excess of 400 °C. The repeat unit of the polymer is:



BPDA-PDA films were prepared by spin-coating the precursor poly(amic acid) dissolved in *N*-methylpyrrolidinone onto polished Si(100) wafers. The poly(amic acid) ester was converted to the polyimide by heating the films under N_2 at 5 °C min⁻¹ to 250 °C, 300 °C or 400 °C and holding at the specific temperature for 3 h. Films with thicknesses of 6 or 200 nm, as determined by ellipsometry, were investigated. The film thickness was varied by changing the concentration of the solution. Rubbing was performed at room temperature (~20 °C) by pressing the polyimide-coated wafer onto a velour cloth under a load of 2 g cm⁻² (200 Pa) and unidirectionally pulling the cloth 300 cm at 1 cm s⁻¹. Only the results for films imidized at 300 °C will be reported here, because they are representative of the data for all samples studied.

The upper two curves in Fig. 2 (traces a and b) show the X-ray scattering for a rubbed 200-nm-thick film, with $\mathbf{Q}$ parallel and perpendicular to the rubbing direction, respectively, where $\alpha > \alpha_c$, that is, the X-rays penetrate the entire film. These data are similar to those reported by others using much thicker specimens¹⁸, and are identical to data from a 200-nm unrubbed film. Thus the rubbing conditions used do not change the bulk structure of the polyimide. The sharp peaks in the scattering profile have been indexed in the figure and arise from the periodic zig-zag of the BPDA-PDA molecules along the chain axis, as shown in the inset. The intensity of these reflections is proportional to the number of BPDA-PDA molecules oriented with their chain axes parallel to $\mathbf{Q}$. Also evident in the data are two broad reflections at 13 and 30 nm⁻¹ which arise primarily from the intermolecular packing of BPDA-PDA segments normal to the chain axis. The scattering profiles (Fig. 2a, b) are independent of the sample rotation and, therefore, there is no preferred

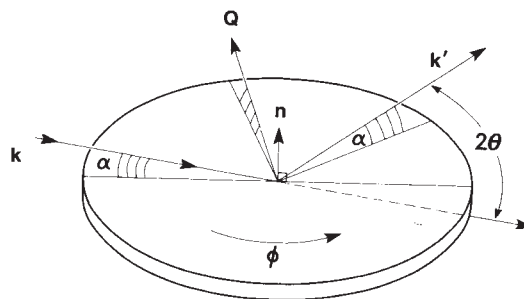


FIG. 1 Grazing-incidence X-ray scattering geometry. The scattering vector $\mathbf{Q}$ is the difference between the scattered and incident X-ray wavevector and has a magnitude of $(4\pi/\lambda) \sin \theta$, where λ is the X-ray wavelength and θ is one-half of the scattering angle 2θ . The surface normal is $\mathbf{n}$ and ϕ describes the rotation angle of $\mathbf{Q}$ with respect to the rubbing direction. The X-ray measurements were conducted on beamline X20C at the National Synchrotron Light Source using a wide band-pass (~1%) synthetic multilayer monochromator.

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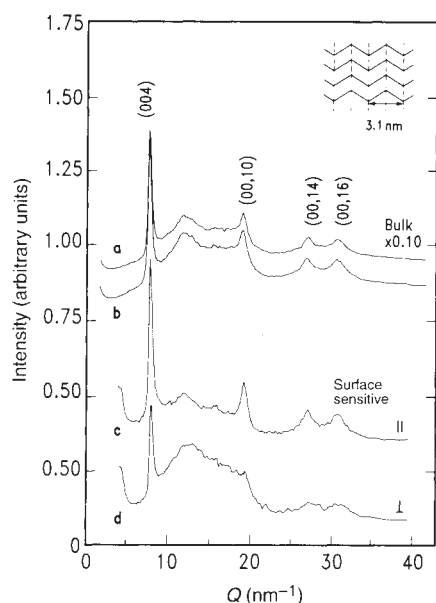


FIG. 2 X-ray scattering intensity (corrected for the illuminated area)²⁴ for a rubbed 200-nm-thick BPDA-PDA film imidized at 300 °C. Trace a, bulk-sensitive scan ($\alpha > \alpha_c$) for Q parallel to the rubbing direction. The labels index the sharp Bragg reflections due to periodic density correlations along the chain axis. Trace b, bulk-sensitive scan ($\alpha > \alpha_c$) for Q perpendicular to the rubbing direction. Trace c, surface-sensitive scan ($\alpha = 0.25\alpha_c$) for Q parallel to the rubbing direction. Trace d, surface-sensitive scan ($\alpha = 0.25\alpha_c$) for Q perpendicular to the rubbing direction. The data have been offset for clarity. Inset, schematic representation of the structural ordering in BPDA-PDA. The dashed lines indicate the periodicity along the chain axis.

alignment of the polymer chains in the bulk of the film under these rubbing conditions.

The lower two scattering profiles in Fig. 2 (traces c and d) were obtained from the near-surface region of the same 200-nm-thick film ($\alpha < \alpha_c$) for Q parallel and perpendicular to the rubbing direction, and are denoted as $I_{\parallel}(Q)$ and $I_{\perp}(Q)$, respectively. Two important differences are immediately evident in comparing these profiles. First, the sharp Bragg reflections arising from the periodicity along the BPDA-PDA molecular axes are significantly more intense for $I_{\parallel}(Q)$ than for $I_{\perp}(Q)$. Second,

the broad reflections at $Q \approx 13$ and 30 nm^{-1} are much stronger for $I_{\perp}(Q)$ than for $I_{\parallel}(Q)$. This result, coupled with the results from the bulk (Fig. 2a, b), shows that only in the near-surface region ($< 5 \text{ nm}$ from the surface) do the BPDA-PDA chains orient along the rubbing direction. Quantitatively, twice as many chains in the surface region are aligned parallel to the rubbing direction than are perpendicular, as measured from the intensities of the (004) reflections. Thus our results suggest that it is the oriented chains at the surface that act as a molecular template in aligning the liquid crystals. From the present measurements it is not known whether the near-surface region consists of a thin layer of chains aligned perfectly along the rubbing direction or of patches of thicker, well aligned domains coexisting with more disordered regions. Measurements of $I(Q)$ and $I_{\perp}(Q)$ as a function of α , which can distinguish between these possibilities, are in progress.

When one considers that the rubbing is done under remarkably small loads ($\sim 200 \text{ Pa}$) and at a temperature (20°C) that is several hundred degrees below the polyimide T_g , it is surprising that the polyimide chains can be oriented at all. This suggests that the yield stress in the near-surface region of the polymer is less than in bulk, due either to a reduction in the number of molecular-chain entanglements near the surface¹⁹ or to a reduction^{20–22} in the near-surface T_g . Alternatively, friction may cause the local temperature at the surface to become high enough for a sufficient period of time that the chains can reorient, as discussed by Bowden and Tabor²³. Experiments are under way to distinguish between these possibilities.

To investigate the effect of film thickness on the molecular orientation, studies were performed on a 6-nm-thick film of BPDA-PDA also imidized at 300 °C. These measurements were done in a GIXS geometry ($\alpha < \alpha_c$) to minimize the background scattering from the Si(100) substrate. Figure 3a shows the X-ray scattering for Q parallel and perpendicular to the rubbing direction. The absence of any sharp Bragg reflections in the perpendicular profile, and the presence of intense (004) and (00, 10) Bragg reflections in the parallel profile, show that the BPDA-PDA chains are well aligned along the rubbing direction. This is further supported by the strong diffuse reflections at $Q \approx 13$ and 30 nm^{-1} in the perpendicular profile. To quantify further the extent of orientation, the intensity of the (004) reflection was measured as a function of the angle from the rubbing direction and is shown in Fig. 3b. It is seen that nearly all the intensity occurs within $\pm 10^\circ$ of the buffing direction.

As the evanescent wave penetrates most of the 6-nm film, even for $\alpha < \alpha_c$, we conclude that essentially all the BPDA-PDA

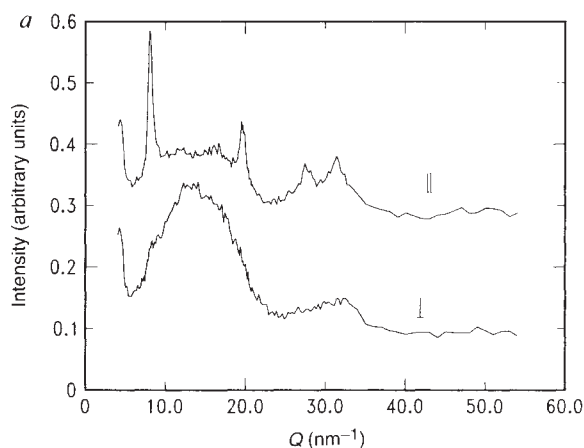
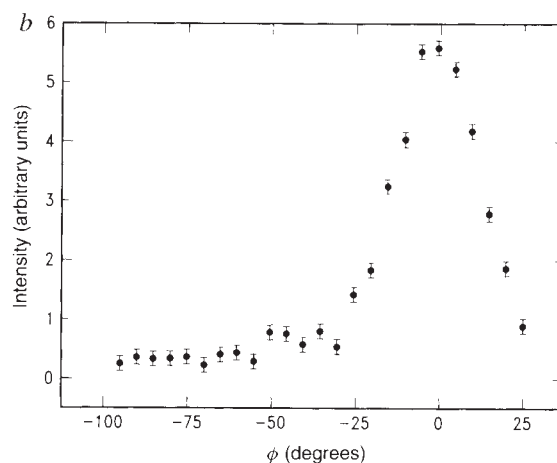


FIG. 3 a, X-ray scattering intensity (corrected for the illuminated area)²⁴ for a 6-nm-thick film of BPDA-PDA imidized at 300 °C. $\alpha = 0.25\alpha_c$ and most of the film is probed by the X-rays. Top trace, Q is parallel to the rubbing direction. Bottom trace, Q is perpendicular to the rubbing direction. b, Intensity of the (004) peak as a function of the angle with



respect to the rubbing direction (ϕ in Fig. 1). The data were recorded with the detector at a fixed 2θ while rotating ϕ . The background, corresponding to the average intensity between $Q = 6.0$ and 8.0 nm^{-1} , was subtracted from the peak intensity.

molecules in the film have been oriented with their chain axes parallel to the rubbing direction. This is in marked contrast to the near-surface (<5 nm) region of the 200-nm film, where the chain alignment was significantly less. This difference shows that one cannot reliably use bulk-sensitive measurements (for example, birefringence) as a function of polyimide thickness to infer surface orientation. In addition, the different responses of the 6-nm and 200-nm films to the identical rubbing conditions indicate that either the T_g or the yield stress of the thinner film is lower than that of the near-surface region of the thicker film. This may be attributed to a reduction in the entanglement density which imparts more mobility to the molecules. □

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Archaean Re–Os age for Siberian eclogites and constraints on Archaean tectonics

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CONSIDERABLE uncertainty surrounds the nature and evolution of the Earth's mantle in Archaean times (>2.5 Gyr ago). Mantle-derived eclogite xenoliths erupted by kimberlites provide important clues in this regard, because their basaltic composition suggests that they may be remnants of an early (>4 Gyr) magma ocean^{1,2}, subducted Archaean oceanic crust^{3–5}, or crystallized high-pressure mantle melts^{6–9}. Better constraints on the age and origin of mantle eclogites are thus important for our understanding of early Earth processes. Here we present rhenium–osmium isotope data for diamond-bearing eclogites from the Udachnaya kimberlite pipe in Siberia, which indicate formation in the Archaean (2.9 ± 0.4 Gyr). This age is too young for the eclogites to be remnants of early Earth differentiation, but overlaps the age range for crust generation and craton stabilization on both the Aldan and Anabar shields of the Siberian craton (2.85–3.2 Gyr)^{10,11}. These data, together with 3.1 Gyr Re–Os model ages for diamond-bearing Udachnaya peridotites¹², show that the Siberian craton lithosphere was at least 150 km thick (the minimum required for diamond stability) by the Mid-Archaean.

A major difficulty with determining the petrogenetic history of mantle eclogites is the lack of definitive age constraints, owing to their complex history^{1,2,4}. Here we use the Re–Os isotope system to determine the age of a well-characterized suite of eclogites from the Udachnaya kimberlite pipe in Yakutia, Siberia¹³.

The large fractionation of Re from Os during mantle melting produces high, variable Re/Os ratios in magmas which should enable the determination of precise model and isochron ages of ancient magmatic products. Furthermore, the Re–Os isotope system appears less sensitive to the widespread lithospheric metasomatism that severely affects incompatible-element-based isotope systems in Siberian xenoliths^{2,12}, and hence provides better prospects for dating. For these reasons we have analysed seven well characterized eclogite xenoliths from Udachnaya, six containing diamonds, with the aim of addressing the age and origin of these rocks.

Re and Os contents of the eclogite suite vary from 0.089 to 1.7 p.p.b. and from 0.028 to 0.346 p.p.b. respectively (Table 1). These abundances and the high ¹⁸⁷Re/¹⁸⁸Os values (~12 to >200) differ from komatiites^{14,15}, a magma type occurring in the Archaean, but span the range of modern^{16–18} and Archaean¹⁵ basalts. Osmium isotopic compositions of the Udachnaya eclogites are highly radiogenic, ¹⁸⁷Os/¹⁸⁸Os = 0.8296 to 9.808, such that between 10 and 55% of the total Os is from ¹⁸⁷Re decay. Thus, Os in the eclogites is considerably more radiogenic than in basalts erupted recently in oceanic settings but is comparable to the present-day isotopic range for magmas erupted in the Archaean (Fig. 1).

To generate the highly radiogenic Os isotopic compositions of the Udachnaya eclogites with their measured Re/Os requires long-term isolation from the convecting mantle. Osmium isotope evolution trajectories for five samples (Fig. 2) show general con-

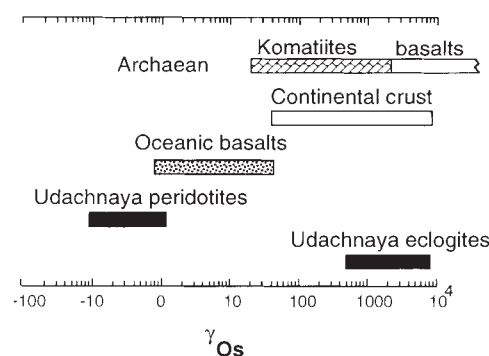


FIG. 1 Osmium isotope composition of Udachnaya eclogites expressed as γ_{Os} . The eclogites are compared to present-day values for Udachnaya peridotites¹², modern oceanic basalts^{16–19}, continental crust²⁸ and Archaean komatiites and basalts^{14,15}.

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TABLE 1 Re–Os isotope systematics of Udachnaya eclogites

Sample	Re (p.p.b.)	Os (p.p.b.)	Os* (p.p.b.)	$^{187}\text{Re}/^{188}\text{Os}$	$^{187}\text{Os}/^{188}\text{Os}$	γ_{Os}	T (Gyr)	ϵ_{Nd}
UV179/86	1.703	0.0880	0.0398	211.1	9.808 ± 16	6759	2.7	
UV179/89	1.634	0.0818	0.0394	209.5	9.141 ± 20	6233	2.6	
U1/79†	0.765	0.0278	0.0207	197.1	3.852 ± 9	2069	1.1	14.1
U5/91	0.813	0.0915	0.0645	63.16	3.761 ± 11	2612	3.4	-20
U281/84	0.391	0.154	0.144	13.37	0.8296 ± 26	501	3.2	6.7
UV464	1.444	0.171	0.124	56.21	3.045 ± 3	2072	3.1	9.5
U236/79	1.538	0.346	0.300	25.03	1.415 ± 2	914	3.1	
U25/84	0.0898	0.0412	0.0372	12.35	1.471 ± 4	1017	6.5	4.3

Whole-rock powders were digested in Carius tubes to overcome problems of spike-sample equilibration³⁰ and analysed for Re–Os isotopes by negative thermal ionization mass spectrometry at the Department of Terrestrial Magnetism using procedures described previously¹². The two replicates of UV179/86 are separate dissolutions, one by Carius tube and one by reducing acid dissolution in a Teflon 'bomb'¹². γ_{Os} values are initial values calculated for a kimberlite eruption age of 350 Myr, where $\gamma_{\text{Os}} = [(^{187}\text{Os}/^{188}\text{Os})_{\text{sample}} / (^{187}\text{Os}/^{188}\text{Os})_{\text{mantle}} - 1]100$; $^{187}\text{Os}/^{188}\text{Os}_{\text{mantle}} = 0.12705$ (ref. 27). Errors on $^{187}\text{Re}/^{188}\text{Os}$ and $^{187}\text{Os}/^{188}\text{Os}$ are estimated at 5% and 2% respectively. Errors on the replicate shown are larger, possibly due to the different dissolution procedure employed or powder inhomogeneity. Model ages are based on derivation from a chondritic reservoir using the relation $T = (1/\lambda) * \ln[(^{187}\text{Os}/^{188}\text{Os})_{\text{mantle}} - (^{187}\text{Os}/^{188}\text{Os})_{\text{sample}} / (^{187}\text{Re}/^{188}\text{Os})_{\text{mantle}} - (^{187}\text{Re}/^{188}\text{Os})_{\text{sample}} + 1]$ where the ^{187}Re decay constant $\lambda = 1.64 \times 10^{-11} \text{ yr}^{-1}$, $(^{187}\text{Os}/^{188}\text{Os})_{\text{mantle}} = 0.12705$ and $(^{187}\text{Re}/^{188}\text{Os})_{\text{mantle}} = 0.3972$. Errors on model ages are generally ± 100 Myr or less.

* Common, non-radiogenic Os.

† Kyanite-bearing sample.

vergence between 2.4 and 2.7 Gyr (Fig. 2) and have Re–Os model ages (see Fig. 2 legend), based on extraction from a chondritic mantle, of between 2.7 and 3.4 Gyr (Table 1). Thus, if the system has remained closed since initial eclogite formation from an assumed chondritic mantle, five of the samples could be between 2.7 and 3.4 Gyr old. Two samples that are clearly divergent on the Os isotope evolution plot give non-Archaeon Re–Os model ages (U1/79, 1.2 Gyr and U25/84, 6.7 Gyr) and have the lowest Os contents of the suite. One, U25/84, has the lowest Re content. These characteristics may have rendered the Re–Os systematics of the samples more susceptible to post-formation metasomatism. Sample UV1/79 contains kyanite. Subsolidus exsolution of kyanite from hyperaluminous clinopyroxene has been proposed as a mechanism for loss of K and Rb from grosspyritic eclogites⁸, and this may have also disturbed both its Re and Os abundances. For these reasons we omitted UV25/84 and U1/79 from regression on a Re–Os isochron plot.

The isochron plot (Fig. 3) defines a correlation with a slope equivalent to an age of 2.90 ± 0.38 Gyr (the 2σ regression error of 0.096 Gyr is viewed as an underestimate due to line fitting control by radiogenic samples, thus all isochron errors are $2\sigma \sqrt{\text{MSWD}}$, where MSWD indicates mean squared weighted deviates). An isochron age is independent of assumptions concerning the derivation of samples. If all the samples except the kyanite-bearing one are included in the regression, the isochron age is still Archaeon but with an enhanced error (2.92 ± 1.1 Gyr). Considering the complex history experienced by these rocks, as evidenced by trace elements¹⁹ and their variable Sr and Nd isotopes², it is remarkable that there is not more scatter on the whole-rock Re–Os isochron diagram. The Os isotope evolution diagram (Fig. 2) suggests that there may have been some variation in initial isotopic composition of the eclogites which could generate scatter on the isochron, but the high $^{187}\text{Re}/^{188}\text{Os}$ of all the samples leads to large uncertainty in the initial ratio. However, an Archaeon age is strongly indicated by the Re–Os data and is supported by a 2.7 Gyr Pb–Pb isochron on clinopyroxene separates from other Udachnaya eclogites⁵. In addition, Os isotope data for a larger suite of diamond-bearing peridotite xenoliths from Udachnaya also give model ages up to 3.2 Gyr¹², within error of the eclogite isochron.

The close correspondence of the Pb–Pb and Re–Os isochron ages for the eclogites and those of peridotite xenoliths from the Udachnaya kimberlite does not support an origin for the eclogites as remnants of the Earth's primary differentiation. Instead, their genesis must be related to craton generation in the Archaeon. Good age determinations for the Siberian crustal basement are scarce. Reliable formation ages for basement in

the Anabar and Aldan shields vary from 2.8 to 3.2 Gyr (refs 10, 11), within error of the lithospheric mantle age documented by the eclogites and peridotites¹². Eclogite formation thus appears to have occurred during the period of crustal formation and craton stabilization. In addition, the 3.2-Gyr Re–Os model ages obtained from diamond-bearing Udachnaya peridotites¹² suggest that eclogite formation could have been synchronous with, or slightly post-dated, the main lithosphere building event, as suggested by the 2.7 to 3.4 Gyr model ages for some samples (Fig. 2). The Archaeon ages for both peridotites and eclogites derived from the diamond stability field indicate that much of the lithosphere sampled by xenoliths in this region (to >150 km depth) had stabilized by Mid- to Late-Archaeon time.

The Archaeon Re–Os age obtained for Udachnaya eclogites agrees with Archaeon ages suggested for some southern African eclogites^{3,4}. These Archaeon ages differ from the Proterozoic model and isochron ages found for eclogite-type minerals included in diamonds^{20,21}, and may indicate that eclogites vary in age and also origin. In fact, several different origins have been proposed for petrographically and geochemically different groups of eclogites from single localities²².

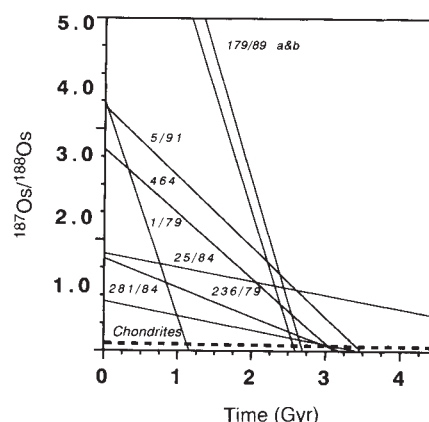


FIG. 2 Osmium isotope evolution diagram for Udachnaya eclogites. Chondrite evolution line from ref. 27. Solid lines define the isotopic evolution of a sample (given by adjacent numbers) through time. A chondritic model age is given by the intersection of the sample isotopic evolution line with that of chondrites (dashed line) and indicates the possible age of a sample, if it originated from a mantle source with chondritic Re–Os isotope characteristics by a single stage event. The numeric derivation of the model age is given in Table 1.

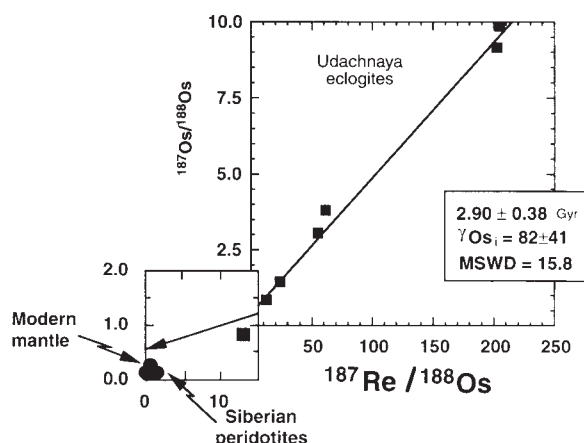


FIG. 3 Re–Os isochron plot of Udachnaya eclogites; error bars are smaller than plot symbols. $\lambda^{187}\text{Re} = 1.64 \times 10^{-11} \text{ yr}^{-1}$, from ref. 29. Samples UV1/79 and UV25/84 are omitted from the plot for reasons stated in the text. See Table 1 for definitions of λ and γOs_1 .

Oxygen isotope characteristics of some diamond-bearing eclogites from Udachnaya are consistent with their protoliths having experienced low-temperature hydrothermal alteration, possibly as oceanic crust⁵. It is also possible that some eclogites crystallized as high-pressure magmatic products in the mantle with no crustal influence^{6–9}. Although the oxygen isotope characteristics of some of the samples studied here are closer to typical mantle values, they show significant variation ($\delta^{18}\text{O} = 4.8\text{--}7.0\text{‰}$)²³ and do not preclude an origin as oceanic crust. Indeed, some Udachnaya eclogites have trace-element concentrations²⁴, including Re and Os, similar to those of mid-ocean-ridge basalts.

The processes involved in generating the elemental and isotopic systematics of the Udachnaya (and other) bi-mineralic eclogites are multi-stage and complex²⁴, rendering it difficult to constrain ages using isotope systems where parent and daughter are large-ion lithophile elements. Indications of Mid- to Late-Archaean ages from Re–Os and Pb–Pb isotope systematics do not favour an origin for these rocks as vestiges of the Earth's primary differentiation in the Hadean (>4 Gyr). Strong arguments have been made for subducted basaltic protoliths for some of these rocks^{6,19}. Although the Re–Os isotope systematics do not provide definitive evidence for a subducted origin, the data do not preclude it, and the very high jadeite contents of the pyroxenes argue against a simple magmatic origin¹⁹. If subduction played a role in the genesis of these rocks then the Re–Os isotope data indicate that it took place in the Archaean.

The crustal basement in the Siberian craton does not appear to be significantly older than the age range seen in the mantle lithosphere defined by Re–Os isotopes¹². This general correspondence between lithospheric mantle age and basement age is similar to that inferred for the Kaapvaal craton^{25,26}, and suggests that either major crust-building and mantle differentiation events are linked by a genetic process, or that survival of voluminous continental crust in the Archaean required the protection of rigid lithospheric mantle 'keels'. □

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Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca*

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MARINE species of *Thioploca* occur over 3,000 km along the continental shelf off Southern Peru and North and Central Chile^{1–4}. These filamentous bacteria live in bundles surrounded by a common sheath and form thick mats on the sea floor under the oxygen-minimum zone in the upwelling region, at between 40 and 280 m water depth. The metabolism of this marine bacterium^{5,6} remained a mystery until long after its discovery^{1,7}. We report here that *Thioploca* cells are able to concentrate nitrate to up to 500 mM in a liquid vacuole that occupies $>80\%$ of the cell volume. Gliding filaments transport this nitrate 5–10 cm down into the sediment and reduce it, with concomitant oxidation of hydrogen sulphide, thereby coupling the nitrogen and sulphur cycles in the sediment.

Thioploca mats, sediment and water chemistry were studied along a shelf transect off the Chilean coast during late summer, March 1994 (Fig. 1). In the densest *Thioploca* mat, at 87 m water depth, the wet weight of sheathed *Thioploca* reached nearly 1 kg m^{-2} , of which the total bacterial biomass accounted for 120 g m^{-2} (wet weight). Single filaments up to 7 cm in length were able to glide into the sediment in sheaths that were 10–15 cm long and 1.5 mm diameter (Fig. 2a, b). Most of the sheaths were horizontally oriented at the sediment surface where they formed yellowish mats up to 2 cm thick, with sheaths

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stretching vertically deep into the sediment (Fig. 3). Up to 100 *Thioploca* filaments, sometimes of two different widths, were observed to share one sheath. Phylogenetic characterization through 16S ribosomal RNA sequencing confirmed that there were two species of *Thioploca*, *T. chileae* (15–20 μm wide) and *T. araucae* (30–40 μm wide), previously described on the basis of filament diameter⁵.

The coastal upwelling zone off central Chile has a very high primary production, 9.6 g carbon $\text{m}^{-2} \text{day}^{-1}$ at the time of our study, among the highest observed in marine environments. Mineralization of the settling organic matter resulted in extensive

oxygen depletion of the water column from the *Thioploca* mat and up to 60 m above the sea floor, with temporal O_2 variations between 0 and 5 μM (Fig. 1). Seasonal oxygen variations are, however, pronounced and strongly influence the mat distribution and biomass (V.A.G., unpublished result). During spring–summer, dense *Thioploca* mats inhabit the sea floor below about 40 m water depth. But the shallower mats break up during winter as bottom-water oxygen increases and nitrate becomes depleted, so dense *Thioploca* mats are observed only at depths below 60 m.

Fine-scale *in situ* measurement of O_2 with microelectrodes mounted on a benthic lander showed no oxygen even in the

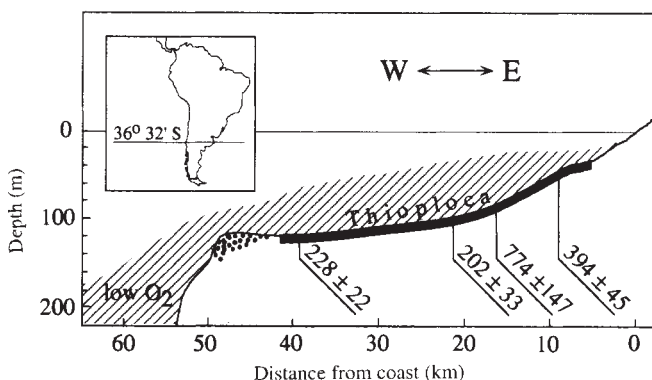


FIG. 1 The continental shelf off Chile at the Bay of Concepción at 36°32' S, studied during late summer 1994 (6 March–1 April). The *Thioploca* mat (black) underlies the oxygen minimum zone (<5 μM O_2 , equivalent to <0.1 ml O_2 litre⁻¹ or <2% air saturation; shaded) in the upwelling region from a water depth of 40 m down to a phosphorite reef on the shelf edge at 120 m. Mat abundance is shown for four stations as wet weight of *Thioploca* filaments plus sheaths ($\text{g m}^{-2} \pm \text{s.d.}$; $n=3$). Undisturbed mat samples of 28 cm^2 were taken by a multiple corer. The mat was gently sieved out of the sediment through a 0.5-mm mesh sieve and infauna and debris were removed before the mat was weighed. From RV *Vidal Gormaz*, the transect was surveyed in a joint research project between the Max Planck Institute for Marine Microbiology, Bremen, Germany, and the Centro-EULA, University of Concepción, Chile.

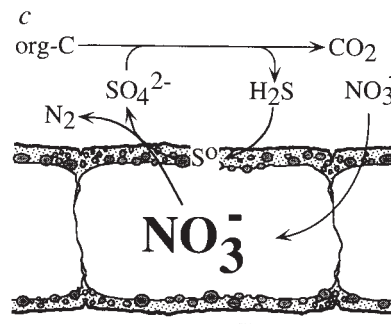
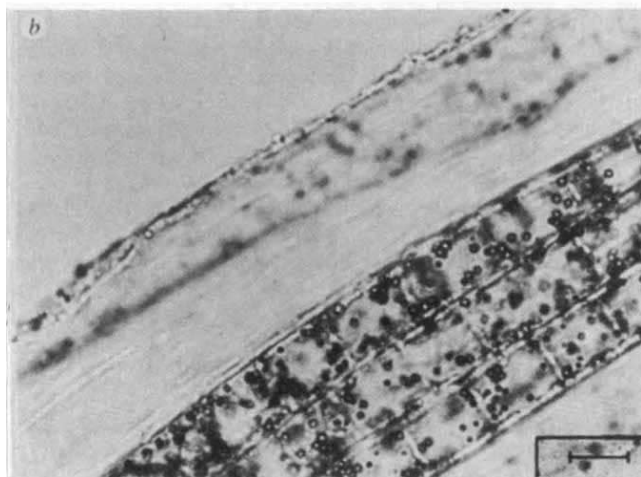
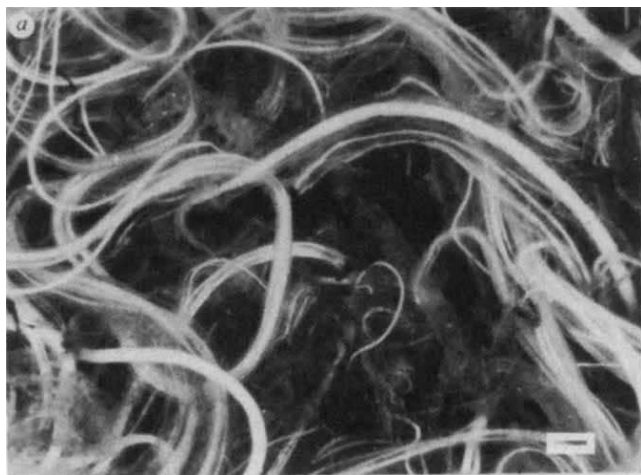


FIG. 2 a, *Thioploca* filaments observed inside sheaths of various thickness and length. The photo was taken after the sediment had gently been washed away from the mat (bar inside white frame represents 1 mm). b, Three multicellular *Thioploca araucae* filaments sharing a common sheath, visible around the filaments as a fibrillar shadow of ~100 μm thickness. Each cell is about 40 μm wide and 60 μm long and filled with light-refracting sulphur globules embedded in the cytoplasm and surrounding a large vacuole (cannot be seen; bar represents 40 μm). c, Schematic drawing of a *Thioploca* cell with elemental sulphur globules (grey) embedded in the cytoplasm (dotted) and the large central liquid vacuole (white) in which nitrate is concentrated up to 500 mM (bar, 40 μm). Nitrate reduction and carbon oxidation are coupled through the sulphur cycle. Hydrogen sulphide is produced by sulphate-reducing bacteria oxidizing organic carbon compounds. *Thioploca* oxidizes hydrogen sulphide using nitrate with concurrent storage of elemental sulphur in the cytoplasm. We suggest the following stoichiometry: $2\text{NO}_3^- + 5\text{HS}^- + 7\text{H}^+ \rightarrow \text{N}_2 + 5\text{S} + 6\text{H}_2\text{O}$, followed by oxidation of elemental sulphur to sulphate: $6\text{NO}_3^- + 5\text{S} + 2\text{H}_2\text{O} \rightarrow 3\text{N}_2 + 5\text{SO}_4^{2-} + 4\text{H}^+$.

top 100 μm of the mat-covered sediment. Organic carbon in the sediment was therefore mineralized entirely through anaerobic processes. Carbon oxidation rates were up to $50 \text{ mmol C m}^{-2} \text{ day}^{-1}$ in the upper 10 cm of the sediment, principally as a result of sulphate reduction. In spite of the production of $25 \text{ mmol H}_2\text{S m}^{-2} \text{ day}^{-1}$, less than $2 \mu\text{M H}_2\text{S}$ was detected in the pore water within this sediment layer. Therefore, the sulphide must have been rapidly reoxidized within the sediment, a surprising result considering the complete absence of oxygen in the sediment. Analysis of nitrate within the cells of *Thioploca*, however, revealed a potential of these bacteria to oxidize part of the sulphide with NO_3^- .

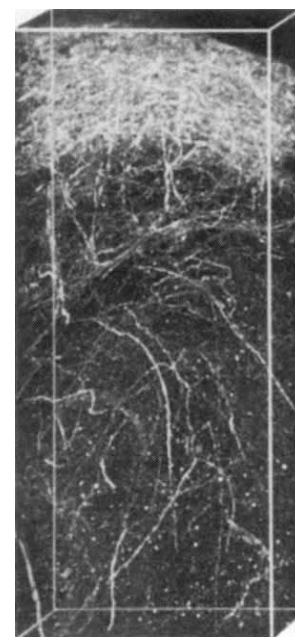
The nitrate concentration in the sea water overlying the *Thioploca* mats was about $25 \mu\text{M}$; in the sediment the concentration decreased to zero at 2–3 cm depth. In contrast, extremely high concentrations of up to 500 mM NO_3^- were measured in clean *Thioploca* filaments collected from the top 6 cm at the same site. *Thioploca* was obviously able to concentrate NO_3^- inside the cell to up to 20,000 times the ambient seawater concentration. To our knowledge, such an extreme nitrate accumulation, comparable to the seawater chloride concentration of 550 mM , has not been observed in any other prokaryote.

A peculiar behaviour and adaptation to this nitrate accumulation by *Thioploca* was demonstrated by addition of nitrate to water overlying *Thioploca* mats in an aquarium with flowing water (flume) under *in situ* conditions. *Thioploca* filaments partly emerged from their sheaths embedded in the sediment and stretched several centimetres up into the sea water after NO_3^- addition. They had the appearance of white hair, swaying in the anoxic or hypoxic water to provide optimal conditions for nitrate uptake.

The large central liquid vacuole, which serves as a store for nitrate, occupies >80% of the cell volume and has been described as an extracytoplasmic reservoir formed by an inflated membrane intrusion⁹. This vacuole is surrounded by numerous sulphur globules embedded in the cytoplasm (Fig. 2b and c). Sulphur globules are also seen in *Beggiatoa*, another mat-forming, filamentous, colourless sulphur bacterium capable of H_2S oxidation with oxygen or nitrate^{10,11}. As shown by 16S rRNA sequencing, *Beggiatoa* is phylogenetically the closest relative to *Thioploca*, sharing many morphological similarities even in its ultrastructure¹².

Beggiatoa and other sulphide-oxidizing sulphur bacteria such as *Thiothrix* spp. and *Thiovulum* spp.^{13,14} depend on the coexistence of their electron donor (H_2S) and acceptor (O_2 or NO_3^-) in order to maintain their energy metabolism and, consequently, they often live in steep, opposed diffusion gradients^{13,15}. Our observations suggest that *Thioploca* is also a sulphide-oxidizing denitrifier. However, by their unique ability to store both nitrate and sulphur in their cells, *Thioploca* become independent of coexisting substrates. By continuous vertical migration between the nitrate-containing sea water and sulphide-producing sediment layers, *Thioploca* can therefore also thrive in sediments at depths where nitrate is lacking. In fact, *Thioploca*

FIG. 3 A computer-generated schematic of a 1.5-cm thick *Thioploca* mat of mainly horizontally oriented sheaths, showing the sheaths stretching more than 6 cm into the sediment. *Thioploca* filaments were observed to move up to 1 cm h^{-1} inside their sheaths and were thus able to transport NO_3^- much faster than diffusion (for example, *Thioploca* filaments may move down to 6 cm within 6 h, whereas diffusion would take $\sim 10 \text{ d}$). Immediately after sampling, the sediment-mat core was frozen in a nitrogen/isopentane slush. The top end of the core liner was tightly closed to allow only a downward expansion of the sediment in order to prevent major changes of the *Thioploca* mat due to swelling. Vertical sections of $100 \mu\text{m}$ thickness were removed by a freeze microtome and after each cut a photo was taken of the remaining core surface in polarized light to enhance the contrast of the sulphur globules. A series of 101 photos were scanned, digitized, and used to construct a 3D image (NIH Image Program) of a 1-cm-thick vertical sediment block. Thus the discontinuity of *Thioploca* filaments at depth is due to their penetration out of the block. The white strands therefore show the orientation of the sulphur-containing filaments, mostly within sheaths.



may outcompete other sulphur bacteria by keeping H_2S and NO_3^- separated.

In oxygen-deficient oceanic waters, carbon oxidation may be coupled directly to nitrate reduction. In the upwelling waters off the Pacific coast of South America, denitrification rates of $25 \times 10^{12} \text{ g N year}^{-1}$ ($3.9 \text{ mmol NO}_3^- \text{ m}^{-2} \text{ day}^{-1}$) have been estimated, comprising up to 25% of the global denitrification in marine water masses^{16,17}. The efficient nitrate uptake and vertical transport by *Thioploca* extends the denitrification zone into the sediment. Carbon oxidation and nitrate reduction are, however, here linked by the sulphur cycle as an intermediate electron carrier (Fig. 2c). Nitrate uptake by a *Thioploca* mat was measured *in situ* as $2.1 \text{ mmol m}^{-2} \text{ day}^{-1}$, which can oxidize up to 20% of the H_2S produced. The *in situ* uptake, however, could be increased by one order of magnitude when seawater nitrate was changed from 20 to $60 \mu\text{M}$. This demonstrates the efficiency by which *Thioploca* filaments are able to take up nitrate. In marine sediments, NO_3^- is normally taken up by diffusion and bio-irrigation when sea water is pumped into tubes and burrows. On the South American Pacific shelf, however, nitrate is transported into the sediment by a previously unknown but unique mechanism, the migration of nitrate-filled *Thioploca* bacteria. □

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Early functional neural networks in the developing retina

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In the adult mammalian retina, the principal direction of information flow is along a vertical pathway from photoreceptors to retinal interneurons to ganglion cells, the output neurons of the retina. We report here, however, that initially in development, at a time when the photoreceptors are not yet even present, there are already functionally defined networks within the retina. These networks are spontaneously active rather than visually driven, and they involve horizontal rather than vertical pathways. By means of optical recording using the calcium-sensitive dye Fura-2, we have found that sets of retinal ganglion cells and amacrine cells, a type of retinal interneuron, undergo synchronized oscillations in intra-

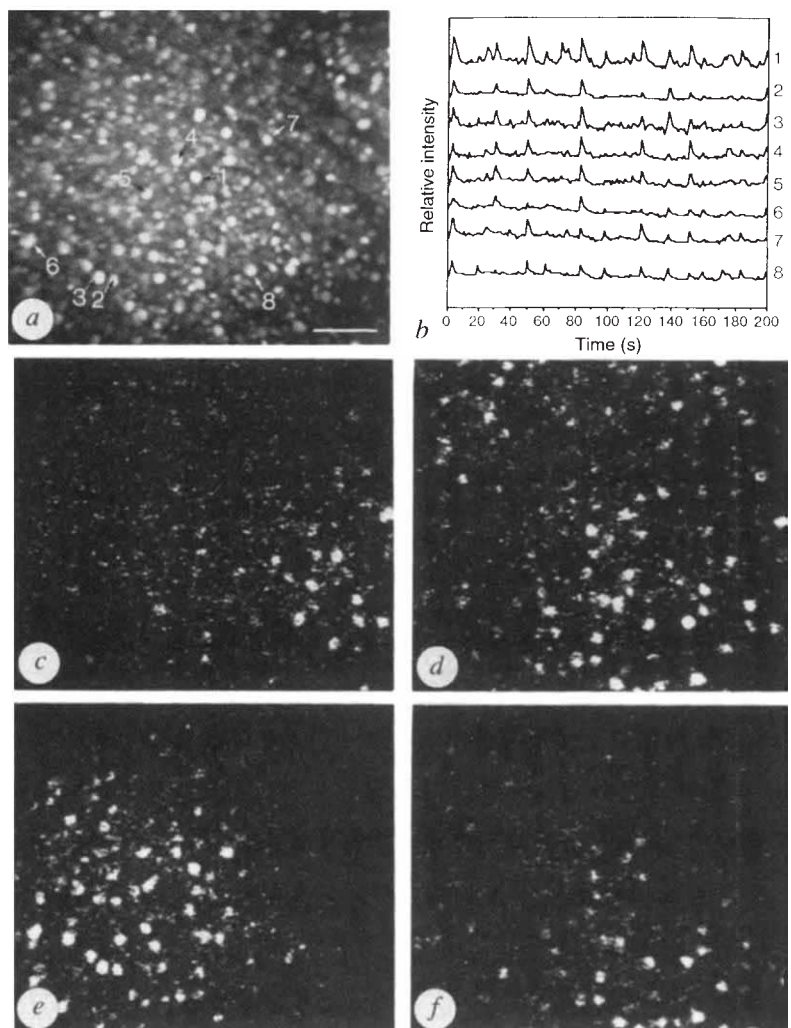
cellular calcium concentration. These oscillations are highly correlated among subgroups of neighbouring cells, and spread in a wave-like fashion tangentially across the retina. Thus, in development of retinal circuitry, the initial patterning of neuronal function occurs in the horizontal domain before the adult pattern of vertical information transfer emerges.

Many cells in the ganglion cell layer (Fig. 1*a*) and the neonatal ferret retina underwent periodic changes in spontaneous internal calcium ion concentration ($[Ca^{2+}]_i$) elevations every 15 s to 90 s (for example, Fig. 1*b*). For each cell, the $[Ca^{2+}]_i$ remained elevated between 10 s and 15 s (thus exceeding the duration of sodium action potential bursts (2 s to 8 s) that were previously recorded using a multielectrode array^{1,2}). Spatial maps of the activity indicate that only a subpopulation of cells showed an increase in $[Ca^{2+}]_i$ during a burst of activity (Fig. 1*c-f*). In some instances, the $[Ca^{2+}]_i$ changes spread across the field of view (Fig. 1*c, d*), in a pattern reminiscent of the wave of action potential activity recorded by the multielectrode array. Sometimes a wave-like spread of activity did not appear during a burst; in these cases (Fig. 1*e, f*) the activity was always seen to be confined to specific areas within the field of view. The location of this 'active zone' varied from burst to burst, but throughout the entire period of recording (up to 2 h), active cells could be found in all parts of the field of view, thus suggesting that there were no physiological or anatomical 'barriers' to the propagation of the activity.

These low-magnification recordings suggest that not all cells within the confines of an active zone undergo changes in $[Ca^{2+}]_i$ during a single burst (compare the number of cells stained with

FIG. 1 Patterns of calcium bursting in neonatal ferret retina. *a*, Ganglion cell layer of a P10 ferret retina viewed at 380 nm excitation after incubation in Fura-2 AM. Scale bar (*a-f*), 100 μ m. *b*, Spontaneous variation in $[Ca^{2+}]_i$ (indicated by changes in fluorescence intensity) of 8 cells from *a* as a function of time; increases in $[Ca^{2+}]_i$ are shown here as upward deflections. *c-f* are difference images (background frame subtracted from a frame in which cells were active), each of which shows only the cells in *a* that were spontaneously active during a time frame of 0.13 s. *c* and *d* were acquired 1 s apart and show a wave-like progression of the activity from the right to left side of the field of view during a burst. *e* and *f* are images from the same field of view showing that different groups of cells can become coactive during two separate bursts, 12 s apart.

METHODS. A total of 16 retinæ from neonatal ferrets, aged postnatal days P4–P12 (when the waves are a robust phenomena), were given an overdose of Nembutal (>50 mg per kg) and their eyes removed. The retinæ were dissected from the eyecups in oxygenated Ames medium (Sigma, 1420) + 20 mM HEPES (Sigma) at 4 °C and held flat by a piece of black filter paper (Millipore, HABP 045). The tissue was incubated in 10 μ M Fura-2 AM, 0.001% pluronic-acid F127 (Molecular Probes) at 32 °C for an hour. Images were captured by a SIT camera (Hamamatsu) during 340 nm and 380 nm excitation, every 1 or 2 s and stored (Panasonic TQ2027). Pharmacological agents were either perfused or pipetted directly into the recording chamber. To display the spatial distribution of 'active' cells, an 8 or 16 frame average was first obtained in segments where $[Ca^{2+}]_i$ was low; this is the background image. An image frame in which cells became active was then subtracted digitally from the background image. The subtracted frame was then coded in red (red channel) and superimposed onto the background frame (composite of blue and green channels) using ADOBE Photoshop 2.0.



Fura-2 in Fig. 1a with the number of active cells seen in c-f). This was confirmed by imaging the retina at higher magnifications which clearly showed that, although many cells were spontaneously active, many others, often small in size, remained silent during a given burst (Fig. 2a). We measured the soma diameters of cells that were either silent or active throughout the entire period of recording (Fig. 2b). The active population consisted of cells with soma diameters that were distributed bimodally; however, mainly relatively small cells contributed to the silent population. The ganglion cell layer of the ferret retina contains both ganglion cells and a subset of amacrine cells whose somata are smaller than those of the ganglion cells and are displaced to this layer^{3,4}. Comparison of the soma diameter plot in Fig. 2b with the known distribution of soma size for displaced amacrine cells and for ganglion cells in P5 ferret (previously identified by retrograde labelling from higher visual centres with horseradish peroxidase (HRP)⁴), suggests that not only ganglion cells, but also some displaced amacrine cells are spontaneously active.

The possibility that amacrine cells, which are generated at the same time as ganglion cells^{5,6}, participate in the correlated activity is supported by the observation that small somata cells at the scleral border of the inner plexiform layer (IPL) are also recruited into this pattern of activity (Fig. 2c). The bursting activity of these cells is correlated with that of cells in the ganglion cell layer (Fig. 2d). However, only an average of $14 \pm 7\%$ (range: 8% to 22%; 242 cells examined from 3 cross-sectional views) of this population of cells participated in the correlated bursting activity. Based on their location and soma size, the spontaneously active cells located in the developing inner nuclear layer are almost certainly amacrine cells, rather than displaced ganglion cells. This conclusion is supported by two other observations. First, we injected fluorescent latex microspheres into the superior colliculi of the ferret neonates and did not observe any retrograde labelling of cells in the inner nuclear layer within the regions where the ganglion cell layer showed many labelled cells (see also ref. 7). Second, our previous observations of injections

of the low-molecular-weight tracer, neurobiotin, into ganglion cells revealed tracer-coupling between ganglion cells and both displaced and normally placed amacrine cells⁸, suggesting that the two cell types are coupled together by intercellular junctions. It is unlikely that other interneurons, such as bipolar cells, represent the active population in the inner nuclear layer because these cells are generated much later in development⁹.

The participation of a subset of amacrine cells in the correlated calcium oscillations suggests that excitation of amacrine cells and ganglion cells may arise through the activation of a neurotransmitter receptor that they both possess early in development. One possibility is that both cell classes are activated by the excitatory amino acid, glutamate, which is present in the developing retina before synapses are even formed^{9,10}. However, although many cells in the neonatal ferret ganglion cell layer were indeed responsive to exogenous application of glutamate, only a subset of these cells were involved in the spontaneously generated bursting activity (Fig. 3a, b). In addition, neither the bursting rhythm of individual cells nor the correlated activity between cells were blocked by the ionotropic glutamate receptor blockers¹¹, CNQX and APV, or the metabotropic glutamate receptor blocker, 1-AP3 (Fig. 3c). Thus, it is unlikely that glutamate neurotransmission mediates the pattern of synchronous bursting activity in this early network. The bursting activity is, however, dependent on sodium channels as no $[Ca^{2+}]_i$ rise was seen in the presence of tetrodotoxin (Fig. 3d).

These findings demonstrate that before the emergence of adult synaptic circuitry, a specific tangential network of ganglion cells and amacrine cells is involved in generating the spontaneous pattern of correlated activity within the developing mammalian retina. This pattern of retinal activity differs from that seen in other systems such as networks of glial cells, epithelial cells or cortical cells, in which the participating cells belong to the same class¹²⁻¹⁷. Furthermore, the results do not support the hypothesis that the spread of activity is due to a nonspecific extracellular diffusion of an excitatory substance, such as glutamate or potas-

FIG. 2 Both ganglion cells and amacrine cells show correlated calcium oscillations. a, c, Spontaneously active cells are shown in red whereas inactive cells are coded in green-blue (see Fig. 1 legend for methods). a, During a burst of activity in this P4 retina, about half the population of cells showed elevated $[Ca^{2+}]_i$. b, Soma diameter histograms of cells shown in a that were spontaneously active compared with histograms of cells that were never active during the entire recording period (160 s, 4 bursts, $n=216$ cells). c, Cross-sectional view of a P10 retina showing spontaneous activity in the ganglion cell layer (GCL, cells 1-5) and in the forming amacrine cell layer (cells 6-10). The approximate boundary between the amacrine cell layer and the ventricular layer (VL) is indicated by arrows. IPL, inner plexiform layer. d, Recordings of spontaneous fluctuations in $[Ca^{2+}]_i$ of the 10 cells marked in c. Scale bars (a, c), 10 μ m.

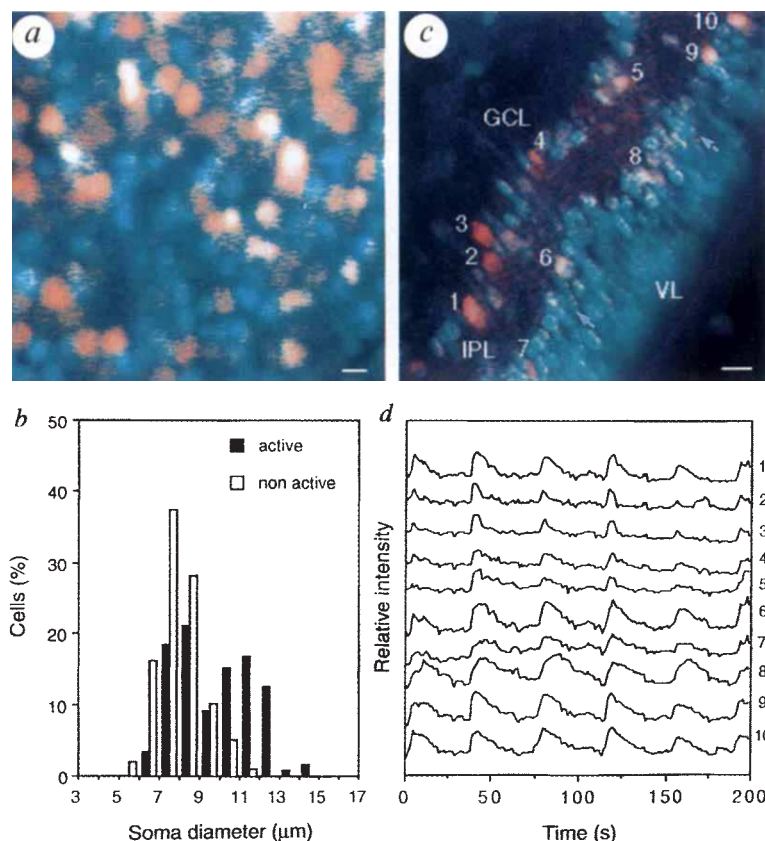
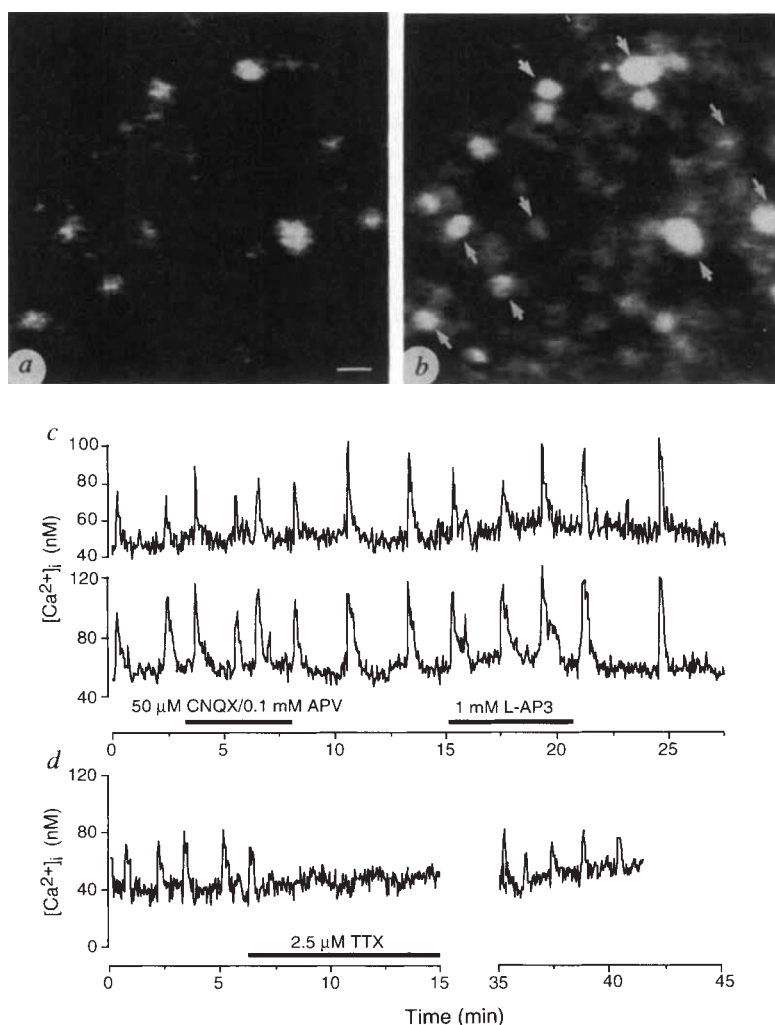


FIG. 3 Correlated bursting activity is not dependent on glutamate receptors, but does require voltage-sensitive sodium channels. *a*, Only a subset of cells in this field of view of the P4 ganglion cell layer were spontaneously active during this particular burst; many others remained inactive until 100 μ M of L-glutamate (Sigma) was added to the bath. Scale bar (*a*), 10 μ m. *b*, Arrows in *b* indicate cells that were spontaneously active in *a*. Each image is the average of 4 video frames (0.13 s recording duration). *c*, Ratiometric measurements, calibrated against known calcium concentrations (see ref. 29 for details), indicate that neither ionotropic nor metabotropic glutamate receptor antagonists block the synchronous elevations in $[Ca^{2+}]_i$. CNQX, 6-cyano-7-nitroquinoxaline-2,3 dione; D,L-APV, D,L-2-amino-5-phosphonopentonic acid; and L-AP3, L-(+)-2-amino-3-phosphonopropionic acid (Tocris Neuramin). *d*, The calcium oscillations were, however, abolished in all cells in the presence of 2.5 μ M tetrodotoxin (TTX; Sigma).



sium, which occurs during spreading depression in other neuronal tissues. Rather, we think it more likely that local release of a neurotransmitter other than glutamate, perhaps in conjunction with gap junctional connections, mediates the initiation and spread of waves in the retina.

What might be the functions of such early networks of cells in which activity is correlated? In the visual system, many lines of evidence suggest that the correlated firing of retinal ganglion cells is required for the formation of precise sets of connections between retinal ganglion cells and their central target structures^{18–22}. The finding here that amacrine cells also partici-

pate in the correlations suggests that specific spatiotemporal patterns of activity may be required for the formation of connections within the retina as well. In addition, the fact that retinal ganglion cells and amacrine cells undergo highly repetitive calcium oscillations over many days raises the possibility that intracellular calcium changes might help drive the growth and further differentiation of these particular cell types^{23–28}. Thus, the correlated calcium bursts in the developing retina may play multiple but highly coordinated roles in the generation of adult neuronal connectivity. □

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A protein related to extracellular matrix proteins deleted in the mouse mutant *reeler*

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THE autosomal recessive mouse mutation *reeler*¹⁻³ leads to impaired motor coordination, tremors and ataxia⁴. Neurons in affected mice fail to reach their correct locations in the developing brain, disrupting the organization of the cerebellar and cerebral cortices and other laminated regions⁵⁻⁸. Here we use a previously characterized *reeler* allele (*rl*¹⁸)⁹ to clone a gene, *reelin*, deleted in two *reeler* alleles. Normal but not mutant mice express *reelin* in embryonic and postnatal neurons during periods of neuronal migration. The encoded protein resembles extracellular matrix proteins involved in cell adhesion. The *reeler* phenotype thus seems to reflect a failure of early events associated with brain lamination which are normally controlled by *reelin*.

The *rl*¹⁸ allele was produced by transgene insertion⁹, providing a probe for the *reeler* locus. To identify candidate genes, we isolated genomic clones using polymerase chain reaction (PCR) primers specific for the 5' region flanking the transgene and screened them for coding sequences by exon trapping¹⁰. A complementary DNA library from adult mouse cerebellum was probed with trapped exons. Using a combination of cDNA library screening, PCR and rapid amplification of cDNA ends (RACE), we obtained a composite full-length nucleotide sequence of *reelin* (Fig. 1).

To confirm that *reelin* is disrupted in *reeler* mutations, we analysed genomic DNA from normal mice or mice homozygous for the two mutations used (*rl* and *rl*¹⁸), using five probes spanning the complete length of *reelin* cDNA (Fig. 2). In samples from *rl* mice, probes 1 and 2 detected bands seen in wild-type DNA, but probe 3 detected a polymorphic restriction fragment, indicative of a genomic alteration near this region. Indeed, the targets for probes 4 and 5 were deleted. A deletion thus appears to have removed about 8 kilobases (kb) of 3' sequence. In *rl*¹⁸, however, although probes 1 and 5 detected bands seen in wild-type DNA, the targets for probes 2, 3 and 4 were absent, indicating that an intragenic deletion has removed between 7.6 and 9.9 kilobase pairs (kbp) of coding sequence. The similar genomic defect in two independent *reeler* strains indicates that the deletions indeed cause the mutant phenotype.

The *reelin* open reading frame is 10,383 bases long, and begins with a methionine codon preceded by a consensus sequence for translation initiation (GGCGGCAUGG)¹¹. The stop codon is followed by about 1 kb of 3' untranslated sequence and a potential polyadenylation signal. It encodes a protein of 3,461 amino acids (Fig. 1a) with a relative molecular mass of 388K. Kyte-Doolittle hydrophobicity analysis reveals an N-terminal signal peptide followed by a consensus cleavage site¹² (Fig. 1a) but no other transmembrane domains, indicating that *reelin* is secreted. The first 500 amino acids are followed by eight consecutive repeats of 350 to 390 amino acids, each containing two related subdomains separated by an EGF-like motif of 30 amino acids (Fig. 1b). The cysteine and tryptophan residues in each subdomain are particularly well conserved. The sequence also contains several potential N-glycosylation, amidation and myristylation sites, and six prokaryotic membrane-lipoprotein-lipid attachment sites. Six of the eight EGF-like motifs contain a consensus (SGxG) sequence for xylosylation¹³ (Fig. 1d). The sequence ends with a short stretch of predominantly basic amino acids.

The amino terminus of *reelin* is 25% identical to that of F-spondin (Fig. 1c), a protein which is secreted from the floor plate of the spinal cord and is thought to regulate the adhesion and extension of commissural axons¹⁴. The N terminus of F-spondin has not been previously implicated in these functions, but its similarity to *reelin* suggests a common role, perhaps in neuronal adhesion. The *reelin* repeats do not match any known sequence, but the EGF-like domains are very similar to each other and are most closely related to those of the extracellular matrix proteins tenascin C, tenascin X, restrictin and the integrin β -chain family (Fig. 1d). As EGF repeats typically occur in regions mediating receptor-ligand interactions¹⁵, the *reelin* repeats may act as a scaffold for other proteins involved in cell adhesion. Thus we suggest that *reelin* is an extracellular protein mediating neuronal adhesion and migration at critical stages of development.

A single *reelin* transcript of about 12 kb was detected in RNA from the brains of normal mice, but not from those of homozygous mutants (Fig. 3a). Intermediate levels were seen in material from heterozygotes of both strains (Fig. 3b). Expression in normal mice was first detected at embryonic day (E) 11.5, preceding the earliest reported defects in *reeler* mice at E14 (ref. 16). Expression increased up to birth (Fig. 3d) and remained high from postnatal days (P) 2 to 11 in both cerebellum and fore/midbrain (Fig. 3c). Expression declined thereafter, and, although traces of messenger RNA were detected in thymus and spleen, adult expression was largely brain specific (Fig. 3a). Expression of *reelin* is thus consistent with its proposed role in neuronal migration.

The principal defect in *reeler* is neuronal malpositioning resulting from abnormal migration^{7,16}. We therefore used *in situ* hybridization to examine *reelin* expression in developing cerebral cortex and cerebellum, two sites strongly affected by the *reeler* mutation. In E13.5 mice (close to the time when the *reeler* phenotype is first detectable¹⁶), *reelin* was expressed throughout the periphery of the telencephalon (Fig. 4a-c). In the cerebral cortex, expression was confined to a continuous single layer of dispersed cells in the marginal zone (Fig. 4b). In the developing hippocampus and olfactory bulb, however, expression was also observed in deeper layers, and lower levels of expression were also detected in the immature striatum. In the cerebellum (Fig. 4d), expression was associated with cells in the rhombic lip, the external neuroepithelium, and an internal zone containing differentiating Purkinje and deep nuclear neurons as well as elements of the cerebellar peduncles. By P10, *reelin* was expressed in the internal granular layer of the cerebellum and the mantle layer of the external granular layer (Fig. 4e). No signal was detected in the cerebellum of *reeler* mice (Fig. 4f).

We have not unambiguously identified the *reelin*-positive cells but their round, light nuclei, evenly dispersed chromatin, and location in the marginal zone suggest they are Cajal-Retzius cells, which are thought to elaborate the extracellular matrix in this area¹⁷. The marginal zone in *reeler* mice appears to contain less extracellular matrix than normal¹⁶, although expression of some extracellular matrix and cell-surface glycoproteins, such as L1, J1 and N-CAM, is unaffected¹⁸. *reelin* may therefore be secreted by Cajal-Retzius cells into the extracellular matrix, where it is required for normal lamination of the cerebral cortex.

Other structures perturbed in *reeler* mice express *reelin*, particularly the hippocampus and the cerebellum⁵ (Fig. 4a, d, e). Expression was observed during hippocampal invagination, and the defects in the embryonic hippocampus may thus reflect perturbations in the earliest phase of development¹⁹. Expression was also observed in the rhombic lip, a region containing migrating neurons and neuroblasts destined to form the olivary nucleus and the granule cell layer of the cerebellum. Interestingly, the olivary nucleus, like the cerebellum, is disorganized in *reeler* mice¹⁶. We did not examine whether Purkinje cells express *reelin* during embryogenesis, although they did not express it at P10 (Fig. 4e). In P10 cerebellum, *reelin* was expressed in a gradient

a

MERGCHAPRA LVLAVLLLA TLARAATGY YPRFSPFFFL CTHHGELECD GEQGEVLISL HIAGNPTYVY PQGEYHVTIS TSTFFDGLLV TGLYTSTSIQ 100

SSQSIGGSSA FGFIMSDHQ FGNQFMCVV ASHVSHLPTT NLSFVWIAPP AGTGCVNFMA TATHRGQVIF KDALAQQLCE QGAPTEATAY SHLAEIHSDS 200

VILRDDFDSDY QQLLEPNINW VECSCNCEME QCGTIMHGNA VTFCEPYGR ELTTTCNLNT TASVLQFSIG SGSCRFPSYSD PSITVSYAKN NTADWQLEK 300

IRAPSNVSTV IHILYLPESA KGESVQFQWK QDSLRVGEVY EACWALDNIL VINSAREVY LEDNLDVDT GNLWFFPGAT VKHSCQSDGN SIYFHNGES 400

EFNFATTRDV DLSTEDIEQW WSEBFESQPT GWDILGAVVG ADCGTVESGL SLVPLKDGGR KLCTPYMDIT GYGNLRFYFV MGCICDPGVS HENDIILYAK 500

IEGRKEHIAL DTLTYSSYKV PSLVSVINP ELQTPATKFC LRQKSHQGVN RNVWAVDFH VLPVLPSTMS HMIQFSINLG CGTHQPGNSV SLEFSTNHGR 600

SWSLHTECL PEICAGPHLP HSTVYSSNY SCWNRITPL PNAALTRDTR IRWRQTGPIL GNMWADINWY IGPSCCLKFCS GRGQCTRHGC KCDPGFSGPA 700

CEMASQTFPM FISESFGSAR LSSYHNFYSI RGAESVFGCG VLAGSKALVF NKDGRRQLIT SFLDSSQSRF LQFTLRGLSK SVLSTCRAPD PQEGGVLLHY 800

SYDNGITWKL LEHYSYVNYH EPRIISVELP DDARQEQIGF RHWQPYHSSQ GEDVWAIDEI VMTSVLFNSI SLDFTNLVEV TQSLGFYLGK VQPYCGHDWT 900

LCFTGDSKLA SSMRYVETQS MQIGASYMIQ FSLVMGCGQK YTPHMDNQVK LEYSANHGLT WHLVQEELP SMPSCQEPFS ASIYHASEFT QWRRVTVLP 1000

QKTWSGATRF RWSQSYTYAQ DEWALDNIYI GQQCPNMCSSG HGSCDHGVCR CDQGYQGTFC HPEALPSTI MSDFENPSSW ESDWQEVIGG EVVKPEQCGG 1100

VVSSGSSLYF SKAGKRQLVS WDLTSSWDF VQFYIIGGE SAACNKPDSR EBGILLQYSN NGGIQWHLA EMYFSDFSKP RFVYLELPA GKTPTCTFRW 1200

WKFVFSGEDY DQWAVDDIII LSEKQKQVIP VVNPTLPQNF YEKPAFDYPM NQMSVWLMLA NEGMKNDSF CATTPSAMVF GKSDGDRFAV TRDLTLKPGY 1300

VLQFKLNIGC TSQFSSTAPV LLQYSHDAGM SWFLKEGCF PASAAGCEG NSRELSEPTV YTGDFEEMT RTIAIPRSL ASSKTRFRWI QESSSQKNVP 1400

FPGLDGVYIS EPCPSYCSGH GDCISGVFC DLGYTAQQT CVSNTPNHSE MDRFEGKLS PLWYKIGCG VGTGCGTLND GRSLYFNLG KREARTVPLD 1500

TRNISLVQFY IQIGKSTSGI TYITPRARYE GLVQVQSDN GILWHLREL DFMSFLEPQI ISIDLPREAK TPATAFRMWQ PQHGKHSQW ALGDLVIGW 1600

DSSQTGFQDK LDGSIDLQAN WYRIQCGQVD IDCLSMIDL IPTENIGNPR YAEWDFHVS ESSFLQWEMN MGCSKPPSGA HGILQLYSLN NGKDWQLVTE 1700

ECVPPTIGCV HYTESSTYS ERFQNMRRVT VYLPLATNSP RTRFRWIQTN YTVGADSWAI DNVILASGCP WMCSSRGICD SGRCVCDRGP GGPFVFPVVP 1800

LPSILKDDPN GNLHPDLWPE VYGAERGNLN GETIKSGTCL IFKGEGLRML ISRLDCLNT MYVQFSRLFI AKGTPERSHS ILLQFVSQGG VTHWLMDEFY 1900

FPQTTRILFI NVLPYGAQT NATRFRWLQF YNNGKKEEII IIDDFIIDGN NLNPLVLLD TDFGPGREDN WFFYPGGNIG LYCPYSSKGA PEEDSAMVFP 2000

SNEVEHSIT TRDLVSNENT IQFEINVC STDSSADVF RLEFSRDEGA TWHLLPLCY HSSSLVSLC STEHPSSTY YAGTTQWRR EVVHFGLHL 2100

CGSVRFWRQY GFYPAGSQPV TWADNVYIG PQCEEMCYGH GSCINGTKCI CDPGYSGPTC KISTKNPDFL KDFEGQLES DRFLMSGGK PSRKGILSS 2200

GNNLFFNEDG LRLMLTRDLD LSHARVQFF MRLGCGKVP DPRSQPVLLQ YSLNGLSWS LLQEFLLSNS SNVGRYALE MPLKARSST RLRWQPSN 2300

GHEFSPWVID QILIGGNISG NTVLEDDFST LDSRKWLHP GGTMPVCSG TGDALVIEK ASTRVVTVD IAVNEDSFLQ IDFAASCSTV DSCYAELEY 2400

SVDLGLSWHP LVRLCLPTNV ECSRYHLQRI LVSDTFNKWT RITLPLPSYT RSQATFRWHQ QAPAPDKQT WADNVYIGD GCLDMCSGHG RCQVGSVCVD 2500

EQWGGGLYCE PETSLEPTQLK DNFNRAPSQ NMLTVSGKL STVCGAVASG LALHFGGCS RLLVTVLNL TNAETQFYF MYGLITPSN RNQGVILLEYS 2600

VNGGITWNL MEIFYDQSK PGFVNILLPP DAKEIATFR WWQPRHGLD QNDWADNVL ISGSADQTV MLDTFSSAPV PQHERSPADA GPVGRIAFEM 2700

FLEDKTSVNE NWLFHDDCTV ERFCDSPDG MLCGSHDRE VYAVTHDLP TENWIMQFKI SVCKVPEKI AQNHQVQFS TDFGVSWSYL VPQCLPADPK 2800

CSGVSQPSV FPPTGKWKRI TYPLPSLTG NVRFRFYQK YSDVQWADN FYLPGCLDN CGGHGDLKE QCICDPGYSG PNCYLTHSLK TFLKERFDE 2900

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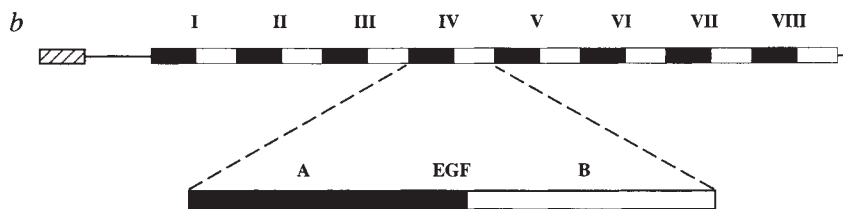
SVRHDYILLP EGALTNTRTL RWWQPFVISN GLVSGVGAC AVGTQSHSDW WSRNHPSQLV DTFDDEGSSH EENWSFYPA VRTAGFCGNP SFHLYWPNK 3100

KDKTHNALSS RELIQPGYM MQPKIVGCE ATSCGLHVS MLEYTKDARS DSWQLVQTC LPSSNSIGC SPFOHEATI YNAVNSSSWK RITQLPDHV 3200

SSSATQFRWI QKGEETEQS WADHVIYGE ACPKLCSSGH YCTTGAVCIC DESFGQDDCS VFSHELPSYI KDNFESARVT EANWETIQGG VIGSGCQOLA 3300

PYAHGDSLYF NGCQIRQAAT KPLDLTRASK IMFVLQIGSP AQTDSCNSDL SGPHADKAV LLQYSVNGI TWHVIAHQHP KDFTQAGRVS YHVPLEARMK 3400

GVLLRWQPR HNGTGHQWA LDHVEVVS TRKQNYMNF SRQHGLRHFY NRRRSLRRY P 3461



A NW[9-12]C[18-26]T.DL.....QF....GC[8-11]V.L.FSD.G.SW.LL...CLP[2-8]C[6-8]S.Y.....W.RIT.LP....S.TFRFW.Q[6-11]WADNVYI
P I Y N T T V V L

B P.LKD.F[7-10]W....GG[5-8]CG[4-7]G.L.F.G.G.R.L.T.DLD....FVQF....G[0-7]C[2-6]P.R.GVLLQYS.NGGITW.LLE.F.....LP.A....TFRFWQ[2-5]G....WAD.I
I E L I F DN Y L V

FIG. 1 Analysis of the predicted amino-acid sequence of reelin. **a**, The reelin amino-acid sequence contains an N-terminal signal peptide (underlined), and eight internal repeats (double-headed arrows I–VIII). Each repeat contains an EGF-like motif (bold). Motif III does not match the consensus perfectly. Amino acids are represented by the single-letter code. **b**, Diagram of the reelin protein structure. reelin contains an N-terminal region of similarity with F-spondin (hatched box), and eight internal repeats of 350–390 amino acids (boxes I–VIII). Each reelin repeat contains two related domains, A (grey) and B (white). The consensus derived for each domain is shown below the diagram. The A and B domains are separated by EGF-like motifs (EGF, black). **c**, Similarity between the N-terminal region of reelin and that of F-spondin. Numbers in parentheses represent the amino acid residues. Identical residues (25%) are shaded. **d**, Similarity between the consensus of the reelin (RL) EGF-like repeats and representative EGF-like motifs of human tenascin (HU TN), human tenascin X (HU TNX), chicken restrictin (GG RES), *Xenopus* (XE) and human (HU) integrin β (ITB) chains 1, 2, 3, 4 and 7, and *Drosophila* Delta (DR DELTA) proteins. One example of an EGF-like motif for each protein is shown. Numbers in parentheses represent the amino-acid residues. Invariable amino acids are shaded.

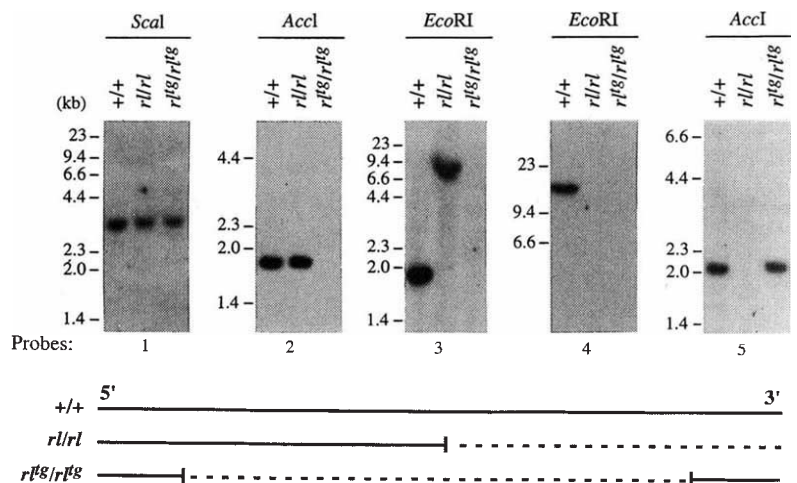
METHODS. Primers derived from the *supfos* transgene 5' flanking sequence were used to screen a P1 mouse genomic library by PCR (Genome Systems). Primer sequences: GD1, CTTCTG-GCCTCTATGGACAC; GD2, TCCATTATTAGGCTGAAGT. The PCR product is 310 bp. The exon trapping strategy¹⁰ was applied to four independent P1 clones using the exon trapping system (Gibco-BRL). To confirm that the trapped DNA fragments were exons, ten independent clones were screened by hybridization with brain RNA. Seven clones, ranging in size from 30 to 240 bp, detected a transcript of

► similar size in RNA from normal mice that was absent in *reeler* mice. The remaining clones did not hybridize to brain RNA. Using the two largest exons as probes, λ ZAP phage clones (Stratagene) were isolated from a cDNA library generated from adult cerebellum and rescued *in vivo*. Initially, we isolated a cDNA clone of approximately 2.3 kbp that contained all of the seven trapped exons. Degenerate primers against the most conserved amino-acid stretches in the reelin internal repeats were used to obtain additional 3' overlapping clones by PCR. 5' RACE PCR was used to obtain the 5' end of the cDNA using a Gibco-BRL kit. PCR fragments were primer tagged and subcloned into a plasmid vector (PCR-Direct cloning system, Clontech). The full-length cDNA sequence is a composite of eight overlapping plasmid clones. The assembly of DNA sequences, conceptual translation, and sequence alignments were performed using the GeneWorks program (Intelligenetics). Amino-acid motifs in reelin were searched in the PROSITE database. Sequence comparison with the GenBank, PIR and SwissProt databases was performed using the BLAST and FASTA network services.

C			
Reelin (1-190)	MERGC---WA PR-ALVLAVL LLATLRARA ATGYPRFSP FFFLCTHHGE	46	
F-spondin (1-190)	MRLSPAPLRL SRGPALLALA LPLAALAFS DET-LDKVAK SEGYSRILR	49	
Reelin (1-190)	LEGDEGEGEV LISLHAGNP TYVVGQBYH VTIST--STF EDGLLVLTGLY	94	
F-spondin (1-190)	AQGTTRREGYT EFSLRVEGDP DFYKPGSSYR VTLSAAPPYS ERGFTLIAL-	98	
Reelin (1-190)	TSTSIQSSQS IGGSSAEGFG IMSDHQFNGQ FMCSVVASHV SHLPTTNLSF	144	
F-spondin (1-190)	-KENREGDKE EDHAGTFQII DEEETQFMSN --CPVAVTES TPRRRRIQV	145	
Reelin (1-190)	VNIAPPAGTG CVNFMATATH RQGVIFKD--ALACQLCEQG APTEATAY	190	
F-spondin (1-190)	FWIAPPTGTG CVILKASIVQ KRIIYFQDEG SLTKKLCEQD PTLDG---	190	
d			
RL EGF-like consensus		G..CP.MCSGHG.C..G.CICD.GY.GP.C	30
HU TN (498-527)	DRQCPDRCNSNRGLCVDGQCVCEDGFTGPD		30
HU TNX (378-407)	QRSCPRGCSQRGRCEGRCVCDPCYTGD		30
GG RES (324-353)	EPRCPRGCSRGVCLGQCVCNDYGGEDC		30
XE ITB1 (601-631)	MAKNGQICNGRGICDCGRCKCTDPKFGPTC		31
HU ITB2 (584-613)	LNPRVECSGRGRRCNVCECHSGYQLPLC		30
HU ITB3 (557-585)	RYKGMCMCSGHGQCSCGDCCLSDWTGYC		30
HU ITB4 (545-574)	PRTSGFLCNDRGRCSMGQCVCEPGWTGPSC		30
HU ITB7 (607-636)	ISPEGLCSGHGRCKNCRCQCLDGYYGALC		30
DR DELTA (422-451)	NCSNPNGINGSCQPSGKCTCPGFSGTRC		30

FIG. 2 The *reelin* gene is partially deleted in mutant *reeler* mice. Southern blot analysis of genomic DNA isolated from normal (+/+) and homozygous mutant mice of the Edinburgh (*rl/rl*) or transgenic (*rl^{ts}/rl^{ts}*) *reeler* strains. DNA was digested with the indicated restriction enzymes and blotted. Each blot was hybridized with the indicated *reelin* cDNA probes. Probe 1, nucleotides 1,196-1,430; probe 2, nucleotides 2,288-2,353; probe 3, nucleotides 3,268-3,613; probe 4, nucleotides 4,620-5,847; probe 5, nucleotides 11,356-11,673. A schematic representation of the extent of the deletion in each mutant *reeler* strain is shown.

METHODS. Genomic DNA was isolated from mouse liver. The DNA (10 μ g per sample) was digested with restriction enzymes, electrophoresed through 0.8% agarose gels, and transferred onto nylon membranes (Hybond, Amersham). Blots were hybridized to *reelin* ³²P-CTP-labelled probes, generated using an oligolabelling kit (Pharmacia). Hybridization was performed under standard conditions, as previously described²⁶.



within the external granular layer (egl) and throughout the internal granule layer (igl). In the egl, *reelin* was associated with postmitotic, premigratory neurons. However, in the igl *reelin* was expressed in postmigratory neurons. Expression persists after neuronal migration is complete in the postnatal cerebral cortex, as well as in the hippocampus and olfactory bulb (Fig. 3c and data not shown), perhaps reflecting a requirement for reelin in stabilizing the cytoarchitecture of the adult brain⁸. Preliminary experiments using glial markers suggest that *reelin* is not expressed in radial or Bergmann glia.

Several lines of evidence strongly suggest that defects in *reelin* are responsible for the *reeler* phenotype. First, *reelin* maps to the *reeler* locus and is partially deleted in two independent mutant strains. Although the 5' end of the *reelin* gene is intact in both strains, no mRNA was detected in either, even with probes from non-deleted regions, indicating that the deletions must either destabilize the transcripts or remove elements required for transcription. Second, exon trapping identified no other transcripts from the *reeler* locus expressed in developing brain. Third, reelin resembles an extracellular matrix protein. This is consistent with the results of studies using chimaeric normal-*reeler* mice suggesting that the *reeler* gene functions extrinsically

to migrating neurons²⁰. Fourth, *reelin* expression is associated with neuronal migration at the time when the first phenotypic defects are detected¹⁶. Formal proof that *reelin* is responsible for the *reeler* phenotype will require complementation of the *reeler* defect using the *reelin* clone.

Although the *reelin* gene has not yet been fully characterized, it appears to be very large, perhaps explaining the frequency with which *reeler* alleles have been identified^{9,21,22}. The exact size of the genomic deletion in *rl* and *rl^{ts}* is unclear, but a 150-kbp deletion in the *reeler* locus of *rl* mice has recently been described², which may correspond to the partial deletion of the *reelin* gene described here.

The gene responsible for a human pathological condition resulting from a neuronal migration defect, Kallmann syndrome, has been identified^{23,24}. Although this gene is unrelated to *reelin*, its protein product has similarities with neuronal cell adhesion and axonal pathfinding molecules. No *reeler*-like phenotype has been reported in humans, but *reelin* mRNA is expressed in human brain, particularly in the cerebellum, and its sequence is highly conserved. The human *reelin* gene maps to 7q22, a chromosomal region not yet linked to any human genetic disease. Given the scarcity of human neurological mutations that

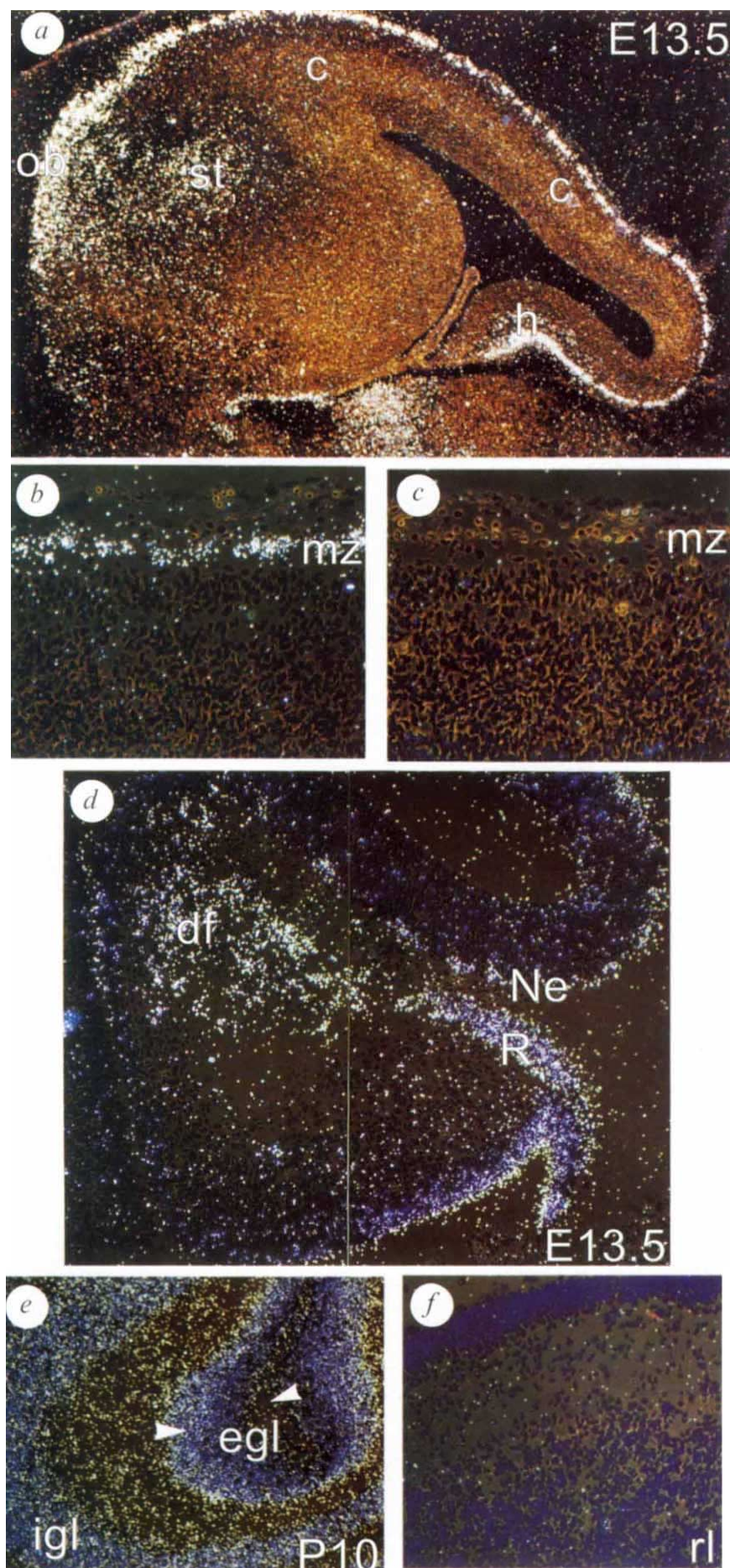
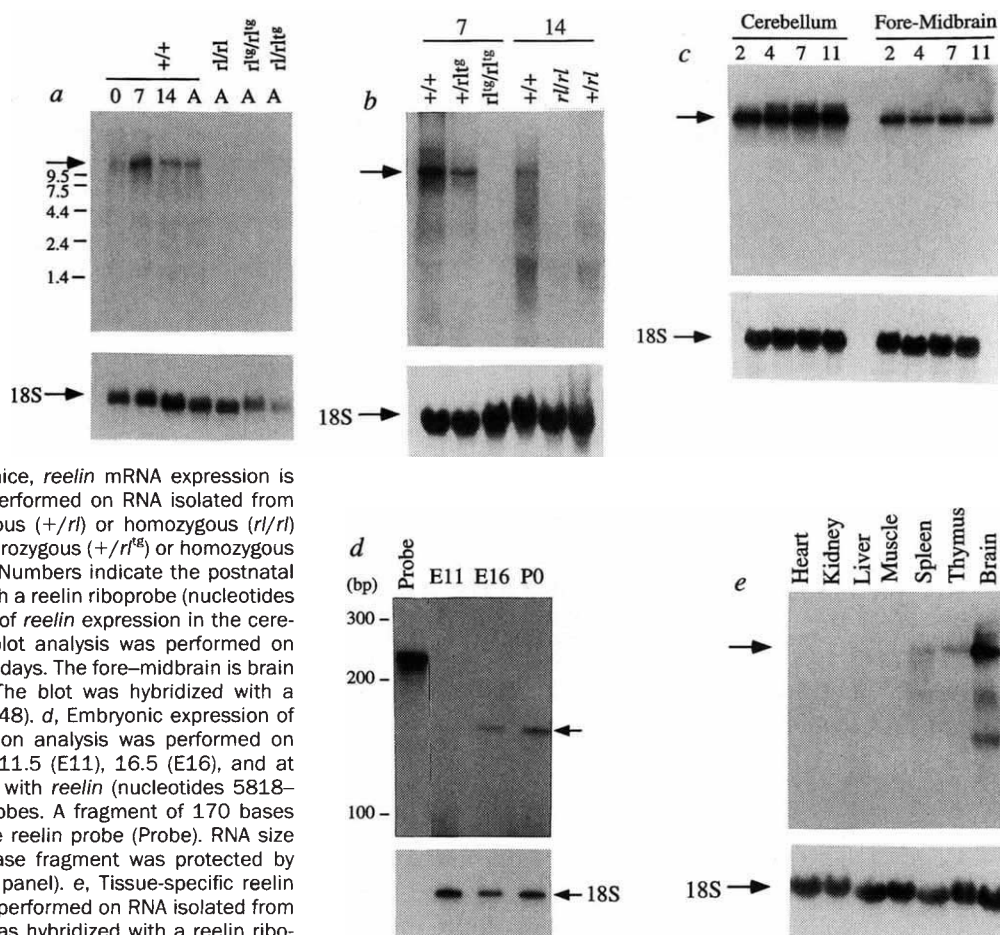


FIG. 4 *In situ* hybridization analysis of *reelin* expression. *a*, Darkfield view of a sagittal section through E13.5 forebrain showing localization of *reelin* mRNA using an antisense probe. Dense accumulation of silver grains was observed within the marginal zone of the cortex (C), the developing olfactory bulb (ob), the hippocampus (h) and the striatum (st). *b*, Higher magnification of E13.5 cerebral cortex showing intense radiolabelling within cells of the marginal zone (mz). *c*, There was no specific labelling using a sense probe as control. *d*, Darkfield view of E13.5 cerebellum showing intense silver grains along the cerebellar rhombic lip (R), the neuroepithelium (Ne) and within differentiating fields (df) that give rise to deep cerebellar nuclei, peduncles and Purkinje cell populations. *e*, Darkfield view of a sagittal section through P10 cerebellum. *Reelin* mRNA was detected in a gradient within the external granule layer (egl) (arrowheads) and throughout the internal granule cell layer (igl). *f*, No expression of *reelin* was observed in P13 *reeler* (rl) cerebellum. In *a*, *b* and *d–f*, sections were hybridized with a *reelin* antisense probe (nucleotides 5818–5973). In *c*, the section was hybridized with a *reelin* sense probe (nucleotides 5714–5844).

METHODS. The tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 and cryoprotected with 20% sucrose in phosphate buffer. Sections (10–14 μ m) were subjected to *in situ* hybridization as described previously^{28,29}. Following hybridization with ³³P-labelled riboprobes, sections were counterstained with 1% toluidine blue in H₂O and mounted with Permount.

FIG. 3 Analysis of *reelin* mRNA expression. **a**, In normal brain, *reelin* mRNA is expressed in a developmentally regulated manner, but it is not expressed in mutant *reeler* brain. Northern blot analysis was performed on RNA isolated from the brain of normal (+/+), homozygous *reeler* of the Edinburgh strain (*rl/rl*), homozygous *reeler* of the transgenic strain (*rl^{tg}/rl^{tg}*), and *reeler* mice heterozygous for each of the mutant alleles (*rl/rl^{tg}*). The brain was dissected from postnatal day 0 to 14, and from adult (A) mice. The blot was hybridized with a *reelin* cDNA probe (nucleotides 1196–1430). A single transcript of approximately 12 kb is detectable (arrow). RNA size markers (Gibco-BRL) are indicated in kilobases. **b**, In heterozygous *reeler* mice, *reelin* mRNA expression is reduced. Northern blot analysis was performed on RNA isolated from the brain of normal (+/+), heterozygous (+/*rl*) or homozygous (*rl/rl*) mice of the Edinburgh strains, and heterozygous (+/*rl^{tg}*) or homozygous (*rl^{tg}/rl^{tg}*) mice of the transgenic strain. Numbers indicate the postnatal age in days. The blot was hybridized with a *reelin* riboprobe (nucleotides 3268–3613). **c**, Postnatal time course of *reelin* expression in the cerebellum and fore-midbrain. Northern blot analysis was performed on RNA isolated at the indicated postnatal days. The fore-midbrain is brain without cerebellum and brain stem. The blot was hybridized with a *reelin* riboprobe (nucleotides 2425–3348). **d**, Embryonic expression of *reelin* in normal mice. RNase protection analysis was performed on head RNA isolated at embryonic day 11.5 (E11), 16.5 (E16), and at birth (P0). The RNA was co-hybridized with *reelin* (nucleotides 5818–5973) and 18S ribosomal RNA riboprobes. A fragment of 170 bases (arrow) was protected by the 220-base *reelin* probe (Probe). RNA size markers (Ambion) are shown. A 70-base fragment was protected by the internal control 18S RNA (bottom panel). **e**, Tissue-specific *reelin* expression. Northern blot analysis was performed on RNA isolated from the indicated adult tissues. The blot was hybridized with a *reelin* riboprobe (nucleotides 3268–3613). In **a**–**c** and **e**, the arrow indicates the ~12-kb *reelin* transcript. Blots were rehybridized with an 18S ribosomal RNA riboprobe to ensure that comparable amounts of RNA were loaded in each lane (bottom panel).



Riboprobes were synthesized using a Promega kit. The *reelin* cDNA probe was synthesized using a Pharmacia oligolabelling kit. ³²P-labeling was performed at 68 °C for riboprobes or 42 °C for oligolabelled probes in standard hybridization buffer containing 50% formamide. For the RNase protection assay, total RNA was isolated using the lithium precipitation method²⁷. The RNA (10 µg per sample) was hybridized at 42 °C with ³²P-UTP-labelled riboprobes specific for *reelin* and the 18S ribosomal RNA. The analysis was performed using the RPA II kit (Ambion). Protected RNA hybrids were electrophoresed through a 5% polyacrylamide-urea gel.

can be tolerated, a null mutation of *reelin* may be lethal. Abnormalities in cortical architecture have been associated with neuronal conditions, including pediatric epilepsy, but the genetic bases for these defects have not been characterized²⁵. It will be of interest to determine whether subtle mutations in *reelin* contribute to these conditions.

Note added in proof: The pattern of *reelin* expression is similar to that of CR-50, a *reeler*-associated antigen that has recently been described³⁰. Thus it is likely that the Cr-50 antibody recognizes a *reelin* epitope. □

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Transformation of axial skeleton due to overexpression of *bmi-1* in transgenic mice

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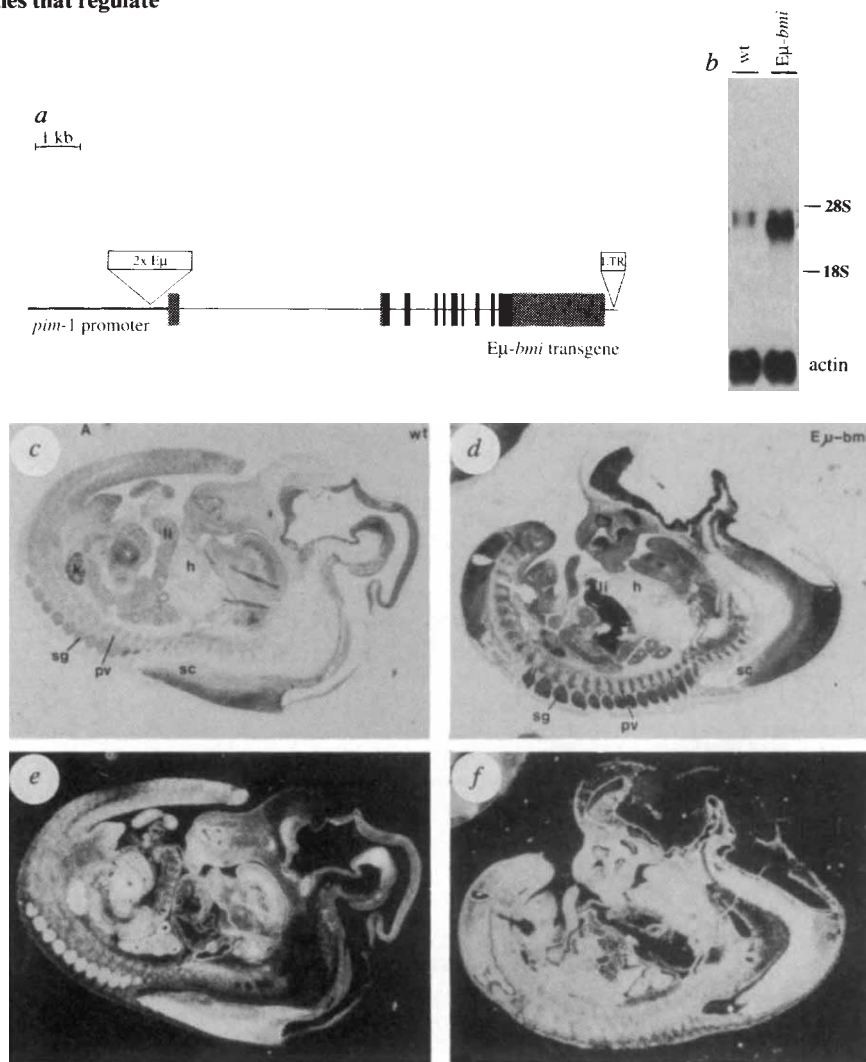
THE oncogene *bmi-1*, which was originally found to be involved in B- and T-cell lymphoma formation¹⁻³ encodes a protein with a domain of homology to the *Drosophila* protein Posterior sex combs (*Psc*) and its relative Suppressor 2 of Zeste (*Su(z)2*) (refs 4 and 5). *Psc* is a member of the *Polycomb*-group gene family, which is required to maintain the repression of homeotic genes that regulate

the identities of *Drosophila* segments⁶. The possibility that *bmi-1* may play a similar role in vertebrates was suggested by our previous finding⁷ that mice lacking the *bmi-1* gene show posterior transformations of the axial skeleton. Here we report that transgenic mice overexpressing *Bmi-1* protein show the opposite phenotype, namely a dose-dependent anterior transformation of vertebral identity. The anterior expression boundary of the *Hoxc-5* gene is shifted in the posterior direction, indicating that *Bmi-1* is involved in the repression of *Hox* genes. We propose that *Bmi-1* is a member of a vertebrate *Polycomb* complex that regulates segmental identity by repressing *Hox* genes throughout development.

We have used a transgenic mouse model system to assess the effect of overexpression of *bmi-1* *in vivo*. The *bmi-1* gene was cloned in a transgene vector linking the gene to the *pim-1* promoter, the immunoglobulin enhancer (*E μ*) and a Moloney murine leukaemia virus (MoMuLV) long terminal repeat (LTR) (Fig. 1a). This promoter and enhancer module gives high levels of transgene expression in lymphoid cells⁸ and is active early in development. Two founder lines, *E μ -bmi 33* and *E μ -bmi 52*, were obtained that transmitted the transgene in a mendelian fashion. Both transgenic lines show a high level of transgene expression in thymus, bone marrow and spleen, and exhibit a high incidence of lymphomas (M.J.A., A.B. and M.v.L., manuscript in preparation). *In situ* RNA hybridization shows that *bmi-1* is

FIG. 1 Structure and expression of the *E μ -bmi* transgene. a, The *bmi-1* sequences were derived from the *bmi-1* genomic clone 6 (ref. 1). Exons are in blocks: solid blocks represent coding regions, and the cross-hatched block represents the 5' and 3' untranslated sequences. The *pim-1* promoter is represented by a thick line; the open boxes represent the inserted immunoglobulin heavy chain and MoMuLV LTR enhancers. b–d, Expression of the *E μ -bmi* gene in transgenic mice at 11.5 d.p.c. b, Northern blot containing total RNA of wild-type (wt) and *E μ -bmi-33* transgenic embryos (11.5 d.p.c.) hybridized with a *bmi-1* cDNA probe. The northern blot was rehybridized with an actin probe to check for the amount of RNA loaded. The 28S and 18S ribosomal bands are indicated. Two endogenous *bmi-1* transcripts¹ are detected in wild-type embryos, whereas an additional highly abundant transgenic transcript of 3 kb is observed in *E μ -bmi* transgenic embryos. c–f, *In situ* RNA hybridization on sagittal sections of a 12.5 d.p.c. wild-type (c, e) and transgenic embryo (d, f). *In situ* hybridization signals appear black in c and d, and white in e and f. Abbreviations: h, heart; k, kidney; li, liver; sg, spinal ganglia; pv, prevertebra; sc, spinal cord.

METHODS. A *Bss*HII/*Xho*I genomic *bmi-1* fragment was cloned in a transgene vector⁸ linking the *bmi-1* gene to the *pim-1* promoter, the immunoglobulin heavy-chain enhancer (*E μ*) and a MoMuLV long terminal repeat (LTR). A purified *Sa*I fragment was used for microinjection into FVB fertilized oocytes as previously described⁸. Transgenic mice and embryos were identified by Southern blotting *Eco*RI digested tail or extra-embryonic tissue DNA, isolated and hybridized as described⁸. The probe used was a *bmi-1* 19.1 genomic fragment¹, labelled by random priming. Male *E μ -bmi-33* transgenic mice were mated overnight with wild-type female FVB mice. Animals that had a vaginal plug at noon the next day were considered as 0.5 d.p.c. Total RNA was isolated by the LiCl-urea method from pooled transgenic embryos and pooled wild-type sibling embryos, Northern blotting and hybridization were performed as described¹. A mouse *bmi-1* cDNA *Bst*XI/*Xho*I fragment was used as a probe. *In situ* hybridization on serial sections using a ³⁵S-labelled antisense RNA probe was performed as described²⁸. A



³⁵S-labelled anti-sense RNA probe was made by *in vitro* transcription with T7 RNA polymerase of a *bmi-1* cDNA *Hpa*II/*Eco*RI fragment. Emulsion-coated sections were exposed for 18 days. After hybridization, sections were stained with haematoxylin.

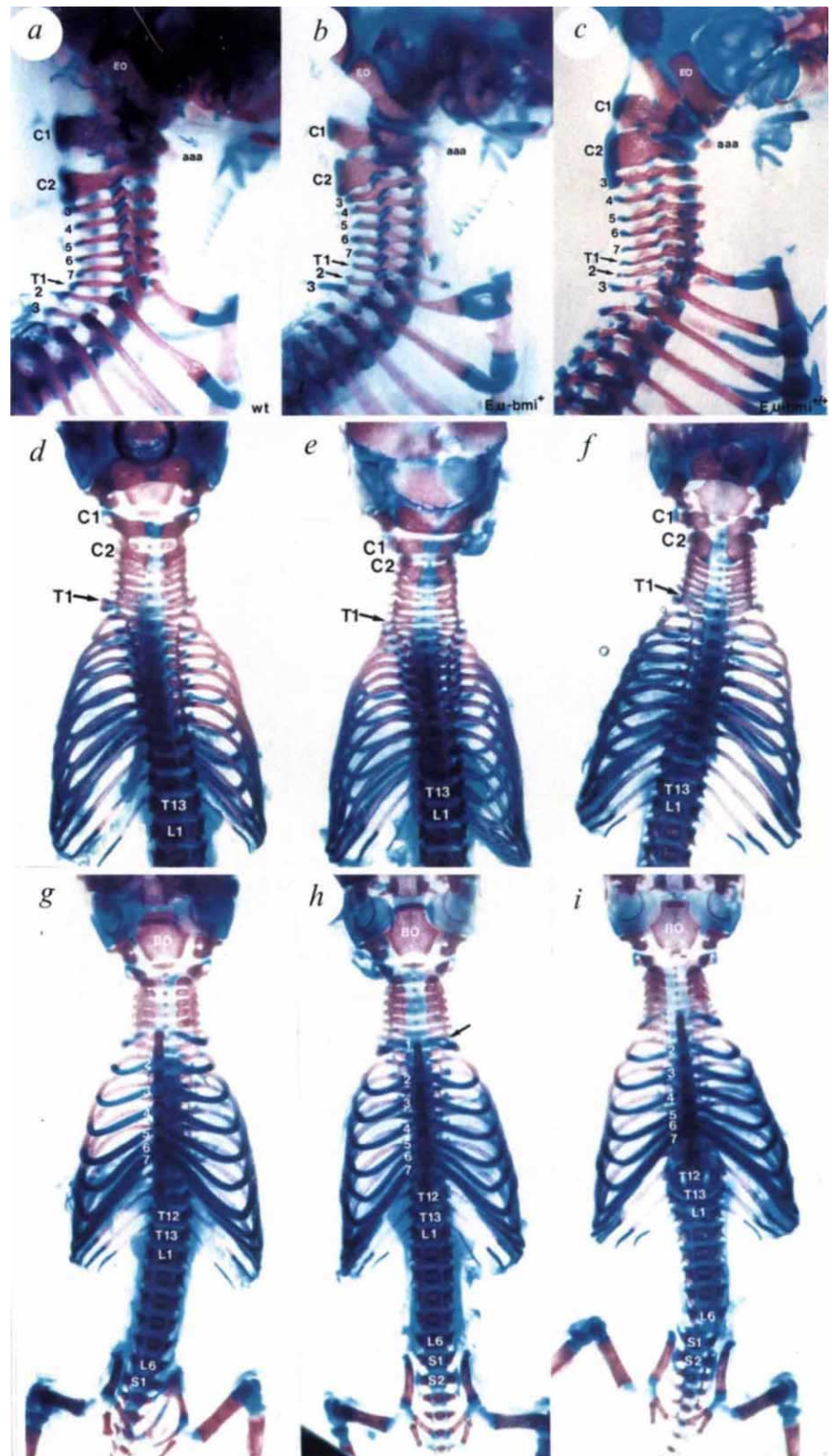
ubiquitously expressed in 12.5-days-post-coitum (d.p.c.) wild-type embryos (Fig. 1c, e), with higher expression levels in brain, spinal cord, spinal ganglia, kidney and lung⁷. A similar expression pattern is observed in transgenic embryos (Fig. 1d, f); the presence of the transgene, however, results in much more *bmi-1* expression (Fig. 1b, f).

Examination of the skeletons of both the $\text{E}\mu\text{-bmi-33}$ and $\text{E}\mu\text{-bmi-52}$ transgenic mice reveals various abnormalities of the vertebral column, none of which is found in wild-type littermates (Table 1). Striking abnormalities are seen in the cervical region

of the transgenic mice (Fig. 2a–f). All of the 19 newborn transgenic mice analysed show thickening of the neural arch of the axis (C2), as observed in *Hoxd-3* (ref. 9) and *Hoxb-4* (ref. 10) nullizygous mice, and can be interpreted as a partial transformation of C2 into the likeness of an atlas (C1). In wild-type mice, the first thoracic vertebra (T1) has a rib that is attached to the sternum (Fig. 2a). In the $\text{E}\mu\text{-bmi}$ transgenic mice, only a residual rib is present on T1 which is no longer connected to the sternum (Fig. 2b). The costal cartilage of the first rib that is connected to the sternum is often split and is now derived from T2. The

FIG. 2. Abnormalities of the axial skeleton in $\text{E}\mu\text{-bmi-33}$ transgenic mice. Whole-mount alizarin red/alcian blue stained skeletons of newborn wild-type (a, d, g), heterozygous (b, e, h) and homozygous (c, f, i) $\text{E}\mu\text{-bmi-33}$ transgenic mice. a–c, Lateral view of the occipital, cervical and upper thoracic region. The numbering C1–C7 and T1–T3 denote the 7 cervical vertebrae and the first 3 thoracic vertebrae. C1 (atlas) with the anterior arch of the atlas (aaa) and a wide neural arch. The neural arch of C2 (axis) is thinner than that of C1 (a). The dorsal cartilage extension, called the processus spinosus, is present on the second thoracic vertebra in wild-type mice (a). The exoccipital bone (EO) and the basioccipital bone (BO) are indicated. Abnormalities in transgenic mice include the thickening of the neural arch of C2 (b, c). In heterozygous transgenic mice a residual rib is present on T1 (b), whereas in homozygous transgenic mice T1 is completely transformed to C7 (c). The costal cartilage of the first vertebrosteral rib is split in heterozygous transgenic mice (b). In heterozygous and homozygous transgenic mice the processus spinosus is present on T3 (b, c). d–f, Dorsal view of the cervical and thoracic region. Note the gene dosage-dependent broadening of the neural arch of C2 in the transgenic mice. On one side the neural arches of C1 and C2 are fused in the homozygous transgenic mouse (f). The arrow indicates the first thoracic vertebra. g–i, Ventral view of the same skeletons as in d–f. In wild-type (g) and the transgenic mice the 7 vertebrosteral ribs are numbered. h, The arrow denotes the split costal cartilage from the first vertebrosteral rib that is derived from T2 in the heterozygous transgenic mouse. A pair of ribs is attached to the first lumbar vertebra (L1) in the transgenic mice (h, i). Note that the rib on L1 is longer in homozygous transgenic mice (i). The first sacral vertebra (S1) is no longer connected to the ilium in the transgenic mice (compare g with h and i) and is transformed to the sixth lumbar vertebra (L6); S2 is the first vertebra that is attached to the ilium. In the homozygous transgenic mice the third sacral is also connected to the ilial bones on one side, indicative of a partial transformation to S1.

METHODS. Whole-mount skeletons of newborn mice were stained with alcian blue 8GS and alizarin red as described⁷. DNA was isolated from a tissue sample and analysed by Southern blot to determine the presence of the transgene as described in Fig. 1. The samples were cleared for 1 week in 1% KOH followed by 1-week clearing steps in 0.8% KOH, 20% glycerol, 0.5% KOH, 50% glycerol. Cleared skeletons were transferred to 50% glycerol, 50% ethanol for photography and storage.



third thoracic vertebra has transformed to the identity of T2, as indicated by the presence of the processus spinosus on T3. The normal number of 7 vertebrosteral ribs, 6 free ribs, 6 lumbar and 4 sacral vertebrae are present in the *Eμ-bmi* transgenic mice (Fig. 2*g-i*). The first pair of vertebrosteral ribs, however, originates from T2, indicating that the ribs of T8 are now attached to the sternum. As shown in Fig. 2*h*, a rib is attached to the first lumbar vertebra (L1). The first sacral vertebra is transformed

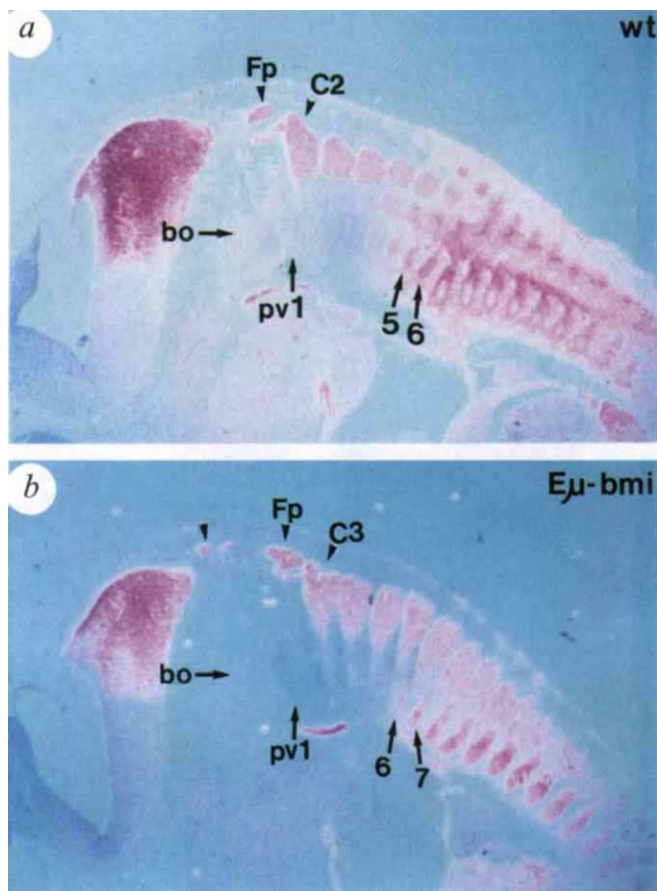


FIG. 3 *Hoxc-5* expression in wild-type and transgenic embryos. Sagittal sections of a 12.5 d.p.c. wild-type (a) and transgenic (b) embryo. Double exposures of bright field, with blue filter, and dark field, with red filter, are shown. In the wild-type embryo (a) the Fp is located anteriorly of the second spinal ganglion (C2). In transgenic embryos C2 is degenerated like the Fp ganglion in control embryos, leaving C3 as the first non-degenerated ganglion. The C2 spinal ganglion is absent in transgenic embryos recovered at 14.5 d.p.c. (data not shown). The remnant of the first spinal ganglion is indicated by a triangle. The arteria vertebralis (visible on slightly parasagittal sections), which, in both transgenic and wild-type embryos, enters prevertebra (pv)6 and ascends through the foramina transversaria of the anterior prevertebra, was used as an independent morphological landmark to assign the identity to the prevertebrae¹². The primordium of the basioccipital bone is indicated (bo). RNA *in situ* hybridization was performed on complete series of sections with a *Hoxc-5*-specific probe. In wild-type embryos at 12.5 d.p.c., pv 6 is the anteriormost prevertebra expressing high levels of *Hoxc-5* mRNA, whereas weak expression is detected in pv 5 (ref. 15) (a). In transgenic embryos the expression boundary has shifted posteriorly to prevertebra 6–7 as no *Hoxc-5* expression is detected in pv 5 and only low levels are present in pv 6 (b).

METHODS. Wild-type (12.5 d.p.c.) and transgenic embryos were obtained and genotyped as described (Fig. 1). Complete series of sections of 3 transgenic and 2 wild-type embryos were hybridized as described²⁸ with a *Hoxc-5* probe¹⁵. A ³⁵S-labelled antisense RNA probe was made by *in vitro* transcription with T7 RNA polymerase of a *Hoxc-5* cDNA *EcoRI/XhoI* fragment. Emulsion-coated sections were exposed for 18 days. After hybridization, sections were stained with haematoxylin.

towards L6 and the second sacral vertebra is attached to the ilial bones, which is indicative of a transformation to S1. Interestingly, the anterior transformation of T8 to T7 and L1 to T13 is also observed in *Hoxc-8* mutant mice¹¹. These findings demonstrate that towards the posterior end of the axial skeleton of *Eμ-bmi* transgenic mice the identity of each vertebra has changed to that of the juxtaposed anterior vertebra. As shown in Fig. 2*c,f,i* and Table 1, the penetrance of the anterior transformation is more pronounced in homozygous *Eμ-bmi-33* transgenic mice.

The partial transformations observed in some mice (Table 1) show that overexpression of *bmi-1* results in anterior transformation along the entire antero-posterior (A-P) axis of the axial skeleton rather than in a change in the number of vertebrae. This phenotype is directly opposite the posterior transformation which is found along the entire A-P axis in *bmi-1*^{-/-} mice⁷. The variable penetrance of posterior transformation is also observed in these mice. Although this could correlate to the hybrid genetic background of the *bmi-1*^{-/-} mice, it probably reflects a stochastic process because the *Eμ-bmi* transgenic mice were of pure FVB inbred origin.

The anterior transformations in *Eμ-bmi* transgenic mice are not restricted to the axial skeleton but also include the spinal ganglia (Fig. 3). In wild-type embryos, the first spinal ganglion (C1), or Froriep's ganglion, degenerates at around day 10.5, leaving a remnant which is still visible at 12.5 d.p.c. (ref. 12) (Fig. 3a). In *Eμ-bmi* transgenic embryos both the C1 and C2 spinal ganglia degenerate (Fig. 3b), as substantiated by the absence of the C2 spinal ganglion in transgenic embryos recovered at 14.5 d.p.c. (data not shown). The degeneration of the second spinal ganglion correlates with the expression of the transgene in the spinal ganglia and can be regarded as an anterior transformation. This phenotype is opposite to the persistence of spinal ganglion C1 in *Hoxb-8* transgenic mice¹³, which can be interpreted as a posterior transformation. Together, our data indicate that *Bmi-1* is implicated in the specification of the identity of the vertebrae as well as the spinal ganglia.

Because *Hox* genes are important in the specification of vertebral identity¹⁴, we examined whether *Hox* gene expression patterns have changed in 12.5 d.p.c. *Eμ-bmi* transgenic embryos. As shown in Fig. 3, in the transgenic embryos the *Hoxc-5* expression boundary has shifted posteriorly from prevertebra 5–6 (ref. 15) to prevertebra 6–7. Our data strongly support a role for *Bmi-1* in *Hox* gene repression, as shown for its *Drosophila* counterpart, *Psc*^{16,17}. That anterior transformations are found over the entire A-P axis of the axial skeleton, in contrast to a limited number of transformed vertebrae in *Hox* loss-of-function mutants^{9–11}, suggests that *Bmi-1* exerts its effect on several *Hox* genes. In this respect the transformations in *Eμ-bmi* transgenic mice resemble the, mainly anterior, transformation along the A-P axis of *RAR-γ* null mutant mice¹⁸. A slight posterior shift of several *Hox* gene expression boundaries, as shown for the *Hoxc-5* gene, would correlate with the subtle shift in vertebra identity.

Polycomb-group (*Pc-G*) loss-of-function mutants in *Drosophila* have no effect on the initial expression pattern of *Hox-c* genes. At later stages of development, however, the homeotic genes are expressed outside their normal domains^{17,19}, resulting in the transformation of the larval segments towards more posterior ones. The *Pc* protein shares a chromodomain with the *Drosophila* heterochromatin protein (HP1), which plays a role in position-effect variegation (PEV)^{6,20}. Together with the genetic interactions¹⁷, colocalization of different *Pc-G* proteins on polytene chromosomes^{21,22}, and direct protein interactions²¹, this has led to a model in which *Pc-G* gene products act in multimeric complexes that stably repress *Hox* gene expression by compacting the chromatin in a condensed inaccessible structure^{6,23}. In agreement with this hypothesis it has been shown that *Enhancer of Zeste* loss-of-function mutants have a decondensed polytene chromosome appearance²², whereas overexpression of *Psc* results in enhanced polytene chromosome condensation²⁴. By

TABLE 1 Abnormalities of the axial skeleton in $E\mu$ -*bmi* transgenic mice

			Wild type (n=17)	$E\mu$ - <i>bmi</i> -33 ¹ (n=14)	$E\mu$ - <i>bmi</i> -52 ⁺ (n=5)	$E\mu$ - <i>bmi</i> -33 ^{+/+} (n=3)
C2	broadened neural arch		0	14	5	3
T1	sternal rib	one side	0	1	1	0
		both sides	17	0	0	0
	rudimentary rib	one side	0	6	3	0
		both sides	0	7	2	0
	no rib	one side	0	5	2	0
		both sides	0	1	0	3
T2	split costal cartilage	one side	0	9	2	0
		both sides	0	4	3	0
T8	ribs attached to sternum		0	14	5	3
L6	pair of ribs		0	14	5	3
S2	connected to the ilium	one side	0	2	1	0
		both sides	0	12	4	3

The table shows that the penetrance of the anterior transformation varies between the different mice analysed and is often asymmetric. Genotype analysis and the preparation of the skeletons was performed as described in Fig. 1. Malformations were scored under a dissecting microscope. The malformations of the axial skeleton were similar for the transgenic mice of the independent $E\mu$ -*bmi*-33 and $E\mu$ -*bmi*-52 lines. The penetrance of the anterior transformation in homozygous $E\mu$ -*bmi*-33 transgenic mice is more pronounced; from T1 towards the posterior end of the skeleton, each vertebra has completely transformed towards the identity of the juxtaposed anterior vertebra.

analogy, we suggest that *Bmi-1* is a component of a murine *Polycomb* complex, which is involved in the maintenance of the repressed state of homeotic genes throughout development. Because *Pc-G* genes are also involved in the regulation of gap genes which act in earlier stages of development²⁵, we cannot yet exclude the possibility that *Bmi-1* is involved in the initiation of *Hox* gene expression patterns. Our experiments provide the first example of overexpression of a *Polycomb*-group gene leading to anterior transformations. Although overexpression of *Psc* in *Drosophila* results in abnormal bristle formation, this phenotype could not be correlated to segmental transformation²⁴. The dosage-dependent anterior transformation in the $E\mu$ -*bmi* transgenic mice is reminiscent of the related phenomenon of PEV. Some enhancer mutations of PEV induce partial homeotic transformations in abdominal segments of adult flies²⁶ and, moreover, mutations in members of the *Pc-G* (ref. 20) and *trithorax*-group (ref. 27) genes can modify PEV. Several modifiers enhance variegation when their gene copy number is duplicated or suppress variegation when deficient^{20,23}. It is suggested that these modifiers code for structural components of heterochromatin that form multimeric protein complexes which assemble in a modular fashion along specific segments of the chromosome. According to a model, based on the law of mass action, a change in the concentration of one of the components affects the concentration of the complex and thereby the extent of heterochromatin spreading²³. Similarly, overexpression of *bmi-1* might result in a murine *Polycomb*-group-induced, chromatin condensation of homeotic loci beyond their normal boundaries, thereby impairing the expression of normally active *Hox* genes. □

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The barley *Hooded* mutation caused by a duplication in a homeobox gene intron

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IN barley (*Hordeum vulgare* L.) the unit of inflorescence is the spikelet, which bears a fertile bract, the lemma, and the floret consisting of palea, two lodicules, three stamens and the pistil¹. The *Hooded* mutation causes the appearance of an extra flower of inverse polarity on the lemma². This phenotype is governed by the single dominant genetic locus *K*³. Here we show that the homeobox gene *Knox3* represents this locus. Ectopic *Knox3* gene expression in the primordium of the extra floret is caused by a 305-base pair duplication in intron 4, and phenocopies of the mutation are obtained in the heterologous tobacco system by *Knox3* overexpression. It is concluded that homeotic genes of the *Knox* gene family are involved in floral evocation. Furthermore, the study of polarity of reproductive organs in *K* and related mutants can now focus on homeobox genes.

In wild-type barley, the lemma is completed by the awn, an appendage homologous to the laminae of normal leaves⁴. In *Hooded* phenotypes (detailed descriptions in refs 2, 5 and 6), periclinal cell divisions in the subepidermal layer of the awn primordium give rise to a meristematic cushion², which differen-

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TABLE 1 Genotype specificity of DNA fragments revealed by (a) RFLP analysis and (b) PCR amplification

	(a) Enzyme/probe combination						(b) Primer combination					
	<i>Rsa</i> /P1 Presence of band a		<i>Rsa</i> /P2 Presence of band		<i>Rsa</i> /P3 Presence of band		A		B		C	
	+	–	b	c	d	e	f	g	h	i	j	k
Number of awned* genotypes tested	44	1†	0	45	3§	38	0	33	0	37	0	6
Number of Hooded† genotypes tested	0	34	34	0	34	0	34	0	3	0	33	1¶

* A total of 70 awned lines were considered, of which 50 were chosen to maximize the genetic distance; these originated in France (16), Germany (14), Italy (9), Great Britain (5), Sweden (3), The Netherlands (2) and Belgium (1). Seeds were provided by A. M. Stanca, Instituto per la Cerealicoltura, Fiorentuola, Italy. A further 14 awned barley varieties originating from Nepal (10), Tibet (3) and India (1) were from the collection of MPI für Züchtungsforschung, Cologne, Germany (accession numbers 4036, 2825, 1679, 1994, 1780, 1776, 2002, 2524, 2358, 2354, 2356, 1984, 2826 and 2532). A further 6 awned genotypes were obtained from the Bundesforschungsanstalt für Landwirtschaft (FA), Braunschweig, Germany. One accession was from the former Soviet Union and is classified as BGRC 39865 *H. spontaneum* (this species is considered the wild progenitor of barley). Five accessions with BGRC numbers 6014, 6016, 39861, 41349 and 41403 were collected in China and in the former Soviet Union and are classified as *H. agriocrithon*, a wild form of *H. vulgare* present in the Himalaya region.

† The 34 Hooded lines included the *K* BGS 152 and the *K^e* lines from the Department of Agronomy, Colorado State University, Fort Collins, USA; the *K* Atlas variety obtained from G. L. Stebbins, Department of Genetics, University of California, Davis, USA; and 31 *K* varieties from the germplasm collection present at the MPI für Züchtungsforschung, Cologne, Germany. Of these, 14 have the BGRC numbers 10203, 5267, 5390, 5439, 5534, 5983, 6002, 6011, 5137, 5002, 4981, 4993 and 5022, and 17 the MPI numbers 2661, 2527, 3011, 23, 1002, 2660, 924, Peru I, 3196, 2037, 29, 27, 2336, 2392, 2352, 2559 and 1014. The origins of these *K* strains are North America (9), Nepal (6), Germany (7), Europe (6), South America (2) and Ethiopia (1).

‡ This corresponds to the awned variety Atem.

§ Awned varieties Atem, Cannon and Zuan.

|| Awned varieties Nudinka, Proctor, Atlas, Gimpel, Foma and Atem.

¶ This represents an *Elevated Hooded* (*K^e*) strain.

tiates the hood consisting of an extra flower with the organs seen in Fig. 1a. Further extra flower primordia may be formed (Fig. 1b), and the hood is inversely oriented with respect to the direction of lemma growth as documented by the change in hair orientation at the hood/lemma border (Fig. 1c). The homeotic mutation of awned (*k*) barley to the Hooded phenotype occurred only once at the end of the eighteenth century in the Himalaya region⁵. An additional allele, *Elevated Hood* (*K^e*), which is dominant to *k* but recessive to *K* and develops a hood more distal on the lemma, has apparently occurred in an unknown cultivar⁷. The dominance of the Hooded mutation and its linkage to an

alcohol dehydrogenase (*Adh*) gene suggested its homology to the *Knotted-1* (*Kn1*) gene of maize. At the *Kn1* locus, only dominant mutant alleles are generated⁸ which induce ectopic malformations (knots) along the lateral veins of the leaf blade⁹. The *Kn1* gene, one member of the *Knox* gene family¹⁰, has been cloned and shown to encode a homeodomain protein¹¹.

Such homeobox-related messenger RNAs were cloned from barley complementary DNA libraries, and the full-length cDNA clone cHvKnox3 encoded a homeodomain protein with 90% amino-acid sequence homology to KN1 (Fig. 2a). The corresponding *Knox3* genes from a Hooded and awned barley variety had an identical structural arrangement (5 introns, 6 exons; Fig. 2b) with introns 2–4 inserted at identical positions in the coding sequence, as are the 4 introns of the *Kn1* gene. The most prominent difference between barley wild-type and mutant *Knox3* alleles was a 305-bp tandem duplication in the large intron 4 of the mutant allele.

The association of *Knox3* sequences to the Hooded phenotype was investigated by restriction-fragment length polymorphism (RFLP) analysis of 45 awned (*kk*) and 33 Hooded (*KK*) lines, as well as of the *Elevated Hood* (*K^e*) mutant and of segregating populations from *K* × *k* crosses. The probes P1, P2 and P3 (Fig. 2b) detected DNA polymorphisms specific for either the Hooded or the awned genotype (Fig. 2c). The only exceptions were the awned varieties Atem, Cannon and Zuan (Table 1), which gave Hooded-specific RFLP patterns. The *Knox3* alleles of these 3 genotypes did not, however, contain the 305-bp duplication in intron 4 revealed by probe P2, indicating that chromosome 4 derived from intragenic recombination events between wild-type and Hooded-like *Knox3* genes. RFLP analysis of F₂ and F₃ plants segregating Hooded and awned phenotypes revealed the absence of recombination between Hooded and *Knox3* (data obtained from 162 segregating chromosomes; not shown). This molecular analysis included *K^e*, and the existence of the 305-bp duplication in this allele (Fig. 2c) provided further support for this insertion being the cause of the mutant phenotypes observed for Hooded genotypes.

These RFLP studies were extended by the polymerase chain reaction (PCR) amplification of the *K*-typical 5' region of intron 4 in the presence of the primer combinations A and B (Fig. 2b, c). A total of 70 awned and 34 Hooded lines were analysed

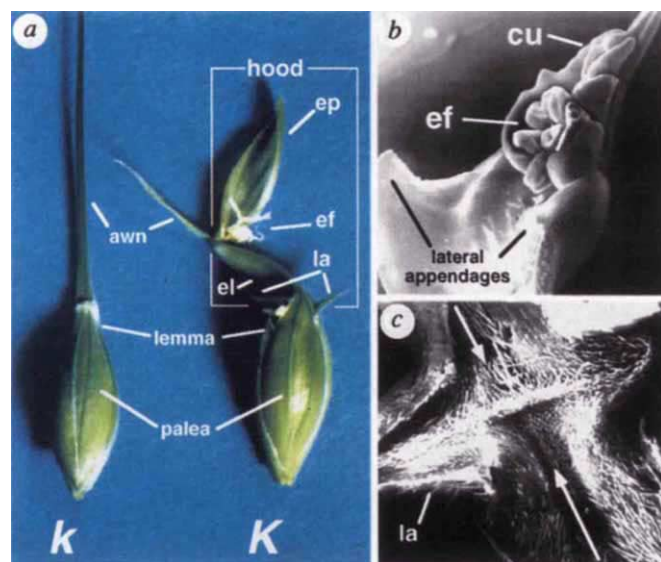


FIG. 1 Phenotype of awned (*k*) and Hooded (*K*) barley. a, Single florets with the hood shown for *K* (ef, extra floret; ep, extra palea; el, extra lemma; la, lateral appendages). b, Scanning electron microscopic (SEM) details of the hood structure with the developed first extra floret (ef) and a second developing extra floret at the cushion stage (cu). c, SEM picture of a *K* floret at the lemma/hood border. Arrows indicate the polarity of the corresponding tissues, as apparent from the direction of growth of hairs.

in this way, and, without exception, in all *K* genotypes (including *K^e*) the PCR fragments had the size expected for the presence of the duplication, and were absent in all awned genotypes (Table 1b; Fig. 2c). For 26 of the *Hooded* lines the PCR product was sequenced, and in every case the duplication was present at the identical site, and its sequence was highly conserved.

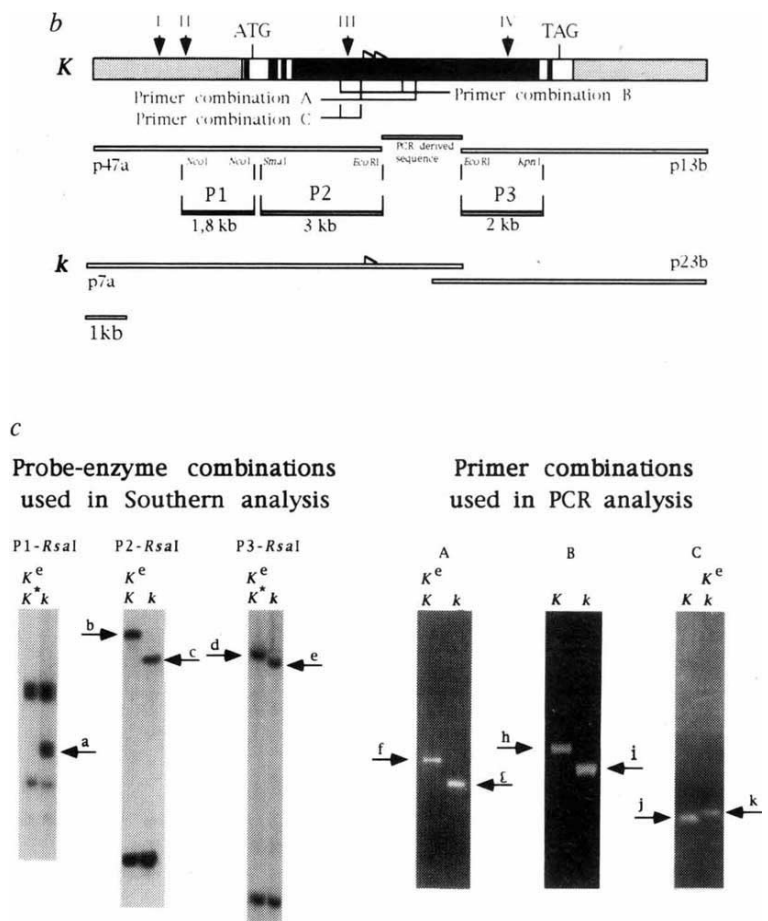
Knox3 gene expression was studied by northern analysis and *in situ* hybridization of barley inflorescences. *Knox3* transcripts had the same size in *K* and *k* genotypes, but were 2.5-fold more

abundant in *Hooded* (Fig. 3a). In flower sections, *Knox3*-specific signals were detected in wild-type (Fig. 3b) and mutant lemmas, and, at lower levels, in the palea and in vascular bundles. A strong hybridization signal was detected at the tip of the *Hooded* lemma before the formation of the cushion (Fig. 3c, d) which, when formed, showed localized *Knox3* overexpression (Fig. 3e). At a later developmental stage, *Knox3* gene expression was downregulated (data not shown). This pattern of gene expression during formation of the epiphyllous flower resembles *Kn1*

FIG. 2 Structure of the barley *Knox3* gene. a, Comparison of the homeobox proteins KNOX3 and KN1. The homeodomains are boxed, and histidine and glutamine stretches are underlined. Arrowheads indicate the position of introns. b, *Knox3* alleles of *K* and *k* genotypes. Introns are boxed black and arrowheads mark sequence polymorphisms. I, 17-bp deletion in *K* and *K^e*; II, 20-bp insertion in *K* and *K^e*; III, 33-bp deletion in *K*; IV, 18-bp insertion in *K* and *K^e*. The triangle denotes the 305-bp sequence of intron 4 duplicated in *K*. The primer combinations A, B and C and the DNA fragments P1, P2 and P3 used in PCR and RFLP analysis are indicated: A covers the 305-bp duplication, B covers this and polymorphism III, and C covers polymorphism III only. c, RFLP and PCR polymorphisms of *Knox3* alleles. Polymorphisms are displayed by the appropriate genotype-specific bands for *Hooded* (*K*), *Elevated Hood* (*K^e*) and awned (*k*) varieties. *K^{*}*, polymorphic fragments also obtained for the awned varieties Atem, Cannon and Zuan (Table 1a). RFLP bands or PCR products b, c, f and g correspond exclusively to the 305-bp duplication. The sizes of the fragments (in kilobases) are: a, 0.35; b, 1.56; c, 1.26; d, 0.87; e, 0.85; f, 1.3; g, 1.0; h, 1.5; i, 1.2; j, 0.47; and k, 0.5.

METHODS. The cHvKnox3 clone was isolated from a λ EM1149 cDNA library prepared from cushion-stage flowers of *K* Atlas by hybridization with a cDNA homeobox clone which was selected from the same library using a maize homeobox-specific ³²P-labelled 51-mer. The sequence for the *Knox3* alleles (*k* allele: accession number X83518) was assembled by sequencing pBluescript subclones according to the chain-termination method in *Taq* DNA polymerase cycle sequencing reactions; p47a, p13b, p7a and p23b correspond to pBluescript subclones of genomic DNA from the *Hooded* variety *K* Atlas and the awned (*k*) variety C1. The original λ EMBL4 clones were isolated by screening genomic libraries of the two varieties with the cHvKnox3 clone. The internal part of intron 4 from *K* Atlas was isolated by PCR amplification of genomic DNA. Polymorphisms were revealed by the probes P1–P3 in Southern analysis of *Rsa*I-restricted genomic DNAs with primer combinations A–C. Additional PCR and sequencing data (not shown) correlated polymorphism IV (Fig. 2b) to the one revealed by the P3–*Rsa*I combination and discovered *K*-specific sequences at sites I and II (Fig. 2b) to be present also in the awned variety Atem. The absence of the awned-specific fragment a in Atem as well as in *K* and *K^e* was related to the loss of an *Rsa*I site by a point mutation.

a	1	50
	KN1 MEEITQHFV GASSHGHG GHHHHHHHH PWASS.LSAV VAPLPFPFP.	
	HvKNOX3 MEEIGHHFGL GATAHGQ... ..HHSQL PWGSSPLSAV ISPPFQQQQQ	
	51	100
	KN1 ...SAGL...PLTLNTVA AT...GNSGG SGNPVLQLAN GGGLLDACVK	
	HvKNOX3 HQQQSAGYLA HSPLSLNTAP PGVSHGGGSG CSNPVLQLAN .GSLLEACAK	
	101	150
	KN1 AKEPSSSSPY AGDVEAIKAK IISHPHYSSL LTAYLECNKV GAPPEVSARL	
	HvKNOX3 AAKEPSSSSY AADVEAIKAK IISHPHYSSL LAAYLDQKV GAPPEVSARL	
	151	200
	KN1 TEIAQVEAR QRTALGGLAA ATEPELDQFM EAYHEMLVKF REELTRPLQE	
	HvKNOX3 TAVAQDLELR QRTALGGLGT ATEPELDQFM EAYHEMLVKY REELTRPLQE	
	201	250
	KN1 AMEPMRRVES QLNSLSISGR SLRNILSSGS SEEDQEGSGG ETELPEDDAH	
	HvKNOX3 AMEFLRRVET QLNSLSISGR SLRNILSTGS SEEDQEGSGG ETELPEDDAH	
	251	300
	KN1 GVDQELKHHL LKKYSGYLSS LKQELSKKKK KGKLPKEARQ QLLSWWDQHY	
	HvKNOX3 GVDQELKHHL LKKYSGYLSS LKQELSKKKK KGKLPKEARQ QLLSWWEMHY	
	301	350
	KN1 KWYPFSETQK VALAESTGLD LKQINNWFIN QRKRHWKPTD EMHHLMDGY	
	HvKNOX3 KWYPFSESQK VALAESTGLD LKQINNWFIN QRKRHWKPTD EMQFVMMDAY	
	351	374
	KN1 HTTN.AFYMD GHFINDGGLY RLG*	
	HvKNOX3 HPPNAAFYMD GHFVNDGSLY RFG*	



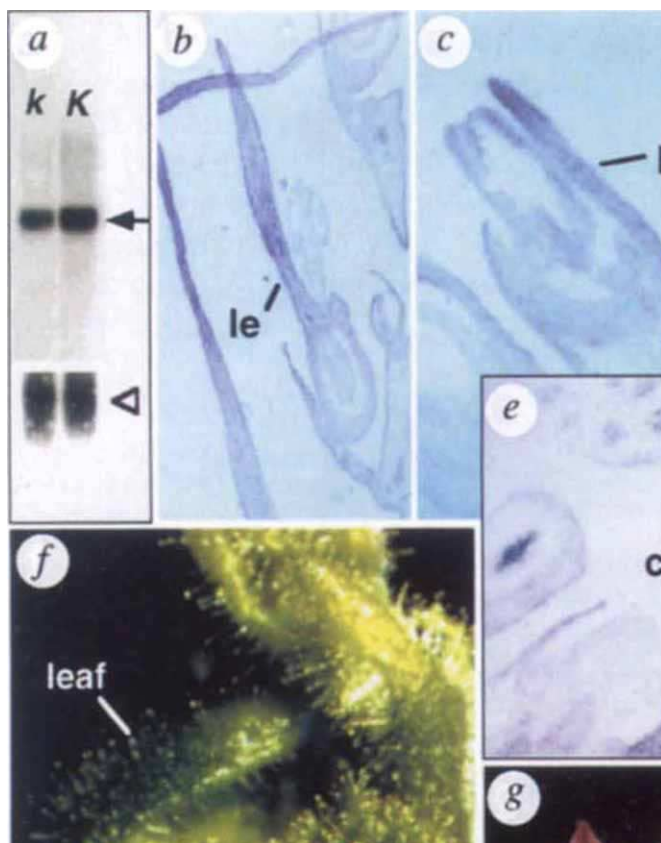


FIG. 3 Homologous and heterologous expression of the *Kn3* gene. *a*, Northern analysis²³ of mRNA from *k* and *K* inflorescences. Arrowhead: 1.8-kb *Kn3* mRNA. *b–e*, *In situ* hybridization of *K* and *k* floret sections. Awned (*b*) and Hooded (*c*) florets are shown at stages preceding, in *K*, the hood formation. Signals are visible for both genotypes predominantly in the lemma (*le*) with the tip of the Hooded lemma showing stronger signals. Sections of Hooded lemmas at the cushion (*cu*) stage hybridized with sense (*d*) and antisense (*e*) probes are shown. *f* and *g*, Phenotype of *chvKn3*-transgenic tobacco type 2 transformants. *f*, An epiphyllous flower on a rudimentary leaf (details of this flower are visible in the SEM insert). *g*, Upper portion of the petals with ectopic outgrowths (*pa*, petal appendages) from a transgenic flower. The insert shows a view from above on this flower.

METHODS. Messenger RNAs were collected at stages corresponding to the cushion stage of the Hooded mutant and analysed by hybridization with a 0.8-kb *chvKn3*-specific *SacI*-fragment (excluding homeobox sequences). Amounts of mRNA were standardized by reprobing with a maize ubiquitin probe (open triangle). For *in situ* hybridization, inflorescences were fixed in 0.1 M phosphate buffer (pH 6.8, 4% formaldehyde, 0.1% glutaraldehyde), embedded in paraffin, and 10- μ m sections were taken. Sections were processed according to standard protocols²⁴ and hybridized with digoxigenin (DIG)-labelled sense and antisense RNA probes representing the 5' 330 bp of *chvKn3*. Hybridized RNA was detected by anti-DIG-sera/phosphatase conjugate binding and reaction with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Transgenic tobacco plants were obtained by leaf disc transformation with the *Agrobacterium tumefaciens* strain LBA4404 carrying a *chvKn3*/pBIN19 construct assembled by cloning the full-length cDNA in 5'-3' orientation into the *EcoRI* site of pRT101 (ref. 25) between the cauliflower mosaic virus 35S promoter and terminator and followed by the insertion of the *HindIII*-released promoter/*chvKn3*/terminator fragment into the *HindIII* site of pBIN19 (ref. 26).

regulation, which is predominantly expressed in the apical meristem, although it is down regulated with the initiation of leaf and flower primordia⁹.

The morphogenetic consequences of ectopic *Kn3* gene expression were evaluated in the heterologous tobacco system by CaMV 35S promoter-driven *Kn3* overexpression in transgenic plants. Two phenotypic groups were obtained. Type 1 plants (5 independent transformants with one copy of the construct) had short leaf blades with an early divergence of lateral veins from the midrib, and resembled the CaMV 35S promoter-regulated *Kn1* cDNA tobacco transformants of the 'rumpled' category¹². Type 2 plants (2 independent transformants with multiple copies inserted) displayed a severely dwarfed phenotype. Basal leaf laminae developed shoots, and upper stem leaves had extremely reduced laminae producing epiphyllous flowers (Fig. 3*f*). Such formation of flowers on leaves is described only for a few genera of the angiosperms^{13,14}, and the barley Hooded mutation is considered to be one example of epiphyllous flower formation^{14,15}. In this sense, the type 2 tobacco transformants reproduce the Hooded phenotype in a heterologous system. In addition, the

petals of every single flower of the main inflorescence showed an unusual phenotype in that they were fused only at the lower part and displayed a tubular shape at the tip (Fig. 3*g*), with ectopic malformations present as outgrowths from the petal margin (*pa* in Fig. 3*g*).

The mechanism by which the 305-bp duplication in intron 4 alters the strength and pattern of *Kn3* expression remains to be determined. Intron 4 sequences downstream of the duplication show remarkable homology to intron sequences of unrelated barley genes^{16,17}, and the enhancement of gene expression due to the presence of introns has been described in genes from mono- and dicotyledonous plants^{18,19}. Furthermore, floral homeotic mutations, such as *ovulata* in *Antirrhinum*²⁰ and *agamous* in *Arabidopsis*²¹, are known to be caused by insertions of DNA sequences into introns of the corresponding genes.

The results reported here bear relevance to the study of flower evocation and polarity of plant organs by focusing on the involvement of homeobox genes that are central to the determination of the basic body structure of several organisms²². □

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Cloning of a *bcl-2* homologue by interaction with adenovirus E1B 19K

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A NUMBER of DNA viruses carry apoptosis-inhibiting genes which enable the virus to escape from the host response¹⁻⁵. The adenovirus E1B 19K protein can inhibit apoptosis induced by E1A, tumour-necrosis factor- α , FAS antigen and nerve growth factor deprivation⁶⁻⁹. The molecular basis of this inhibition remains poorly understood, but the fact that protection is seen in the absence of other viral proteins suggests that E1B 19K targets cellular proteins. We report here the identification of three cellular proteins that bind E1B 19K. One of these is a new member of the *bcl-2* family¹⁰⁻¹⁶, which we have called *bak* (for *bcl-2* homologous antagonist/killer). This protein, which is expressed in a wide variety of cell types, binds to E1B 19K and to the Bcl-2 homologue Bcl-x_L (ref. 17) in yeast. In addition, overexpression of *bak* in

sympathetic neurons deprived of nerve growth factor accelerates apoptosis and blocks the protective effect of co-injected E1B 19K.

To identify E1B 19K binding proteins, we used the yeast two-hybrid system^{18,19} to screen an Epstein-Barr virus (EBV)-transformed human B-cell library and found several interacting clones. These include two independently isolated complementary DNAs encoding large fragments of the lamin A/C protein, in support of an earlier observation that E1B 19K colocalizes with the nuclear lamina in transfected HeLa cells²⁰. The significance of this interaction is unknown but suggests that E1B 19K may participate in the regulation of lamin function. Of the two other clones isolated, the first had no significant homology to any sequences in the nucleotide and protein databases; features of this cDNA will be reported elsewhere. The second clone, *bak*, was found to encode a peptide with extensive sequence homology to the Bcl-2 protein family¹⁰⁻¹⁶.

Subsequent isolation of a larger clone from a JURKAT cDNA library revealed an open reading frame encoding a 216-amino-acid protein with 28% identity to Bcl-2 (Fig. 1a). The homology is particularly striking in the BH1 and BH2 domains of Bcl-2 (Fig. 1b), which are important for Bcl-2 function^{10,16}. Additionally, as in Bcl-2, the C-terminal hydrophobic domain of Bak indicates that it may be membrane-localized. We used a primary cell to investigate the biological activity of *bak*. The full-length cDNA was cloned into the mammalian expression vector pCDNA1amp and microinjected into the nucleus of primary rat sympathetic neurons²¹. These cells die by apoptosis after nerve growth factor (NGF) deprivation, which can be blocked by expression of Bcl-2 and E1B 19K (refs 6, 9). In contrast, *bak* accelerated the death of neurons deprived of NGF (Fig. 2a): 24

a

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-192                                     ACAGGGACAAGT -181
-180 AAAGGCTACATCCAGATGCCGGAATGCAGTACGCCCATCTCGAACTGGGCTCCCA -121
-120 CTCAGCCCTGGGAGCAGCAGCCGAGCCCTCGGACCTCCATCTCCACCTCTCTGAC -61
-60 GCACCCGGTGGGCGAGATCCGCGAGGCTGATCCGCTCTCCACTGAGACCTGAAAA -1

1 ATGGCTTCGGGCAAGGCCAGGCTCTCCAGGAGGAGTGGGAGAGCTGCCCTGCC 60
1 MetAlaSerGlyGlnGlyProGlyProArgGlnGluCysGlyGluProAlaLeuPro 20

61 TCTGCTTCTGAGGAGGAGTACGCCAGGACAGAGGAGGTTTCCGAGCTACGTTTT 120
21 SerAlaSerGluGluGlnValAlaGlnAspThrGluGluValPheArgSerTyrValPhe 40

121 TACCGCATCAGCAGCAAGAGGAGTGAAGGGTGGTGGCCCTGGCGACCCAGAGATG 180
41 TyrArgHisGlnGlnGluGlnGluAlaGluGlyValAlaAlaProAlaAspProGluMet 60

181 GTCACCTTACCTCTGCAACCTAGCAGCAGCATGGGCGAGGTGGGAGGCGAGCTGCCATC 240
61 ValThrLeuProLeuGlnProSerSerThrMetGlyGlnValGlyArgGlnLeuAlaIle 80

241 ATCGGGCAGCAGATCAACCGAGCTATGACTCAGACTTCCAGACCATGTTGGCAGCCTG 300
81 IleGlyAspAspIleAsnArgArgTyrAspSerGluPheGlnThrMetLeuGlnHisLeu 100

301 CAGCCCAAGGAGAGAAATGCCTATGACTACTTACCAAGATTGCCACAGCCTGTTTGA 360
101 GlnProThrAlaGluAsnAlaTyrGluTyrPheThrLysIleAlaThrSerLeuPheGlu 120

361 AGTGGCATCAATTTGGGCGCTGCTGGCTCTTCTGGGCTTCCGCTACGCTCTGGCCCTA 420
121 SerGlyIleAsnTrpGlyArgValValAlaLeuLeuGlyPheGlyTyrArgLeuAlaLeu 140

421 CACGCTTACCAGCATGGCCTCACTGGCTTCTAGCCGAGGTGACCCGCTTCTGCTGCAG 480
141 HisValTyrGlnHisGlyLeuThrGlyPheLeuGlyGlnValThrArgPheValAlaAsp 160

481 TTCATGCTGCATCACTGCATGCCGCGGTGCATTCGACAGAGGGTGGCTGGCAGCCG 540
161 PheMetLeuHisHisCysIleAlaArgTrpIleAlaGlnArgGlyGlyTrpValAlaAla 180

541 CTGAAGTGGGAAATGCTCCCATCTGAACGCTGCTGGTGGTCTGGGTGCTGCTTCTG 600
181 LeuAsnLeuGlyAsnGlyProIleLeuAsnValLeuValValLeuGlyValValLeuLeu 200

601 GGCAGTTTGTGCTACCAAGATCTTCAATCATCACTCCCAAGGTCGCCCTTGGGTCC 660
201 GlyGlnPheValValArgArgPhePheLysSer*** 220

661 CGGTCAGACCCCTGCTGGACTTAAGCGAAGTCTTTCGCTTCTGTTCCCTTGCAGGG 720
721 GTCCCCCTCAAGAGTACAGAGCTTTAGCAAGTGTGCACTCCAGCTTCGAGGGCCCT 780
781 CGCTGGGGGAGTCAAGCTTCAGAGGAGCTCAACATTCAGTGTGCTAGTGGGCGCTCT 840
841 CTTGGGCGAGGGGTGGCGCTCTCTCCCTCAGCTCTCTGGGACCTCTTAAACCTGT 900
901 CCTGCTAGGCGCTGGGAGACTGATACTTGGGAGACAGAGACTGGGACCCACTTCTC 960
961 CCCAGGAAGTGTTAACGGTTTAGCTTTTATAATACCTTGTGAGAGCCATCCAC 1020
1021 CATCTACCTAGGCGAGGAGCTCTGGGCTGGGAGTGGTGGCTATGCTTCCCGAGG 1080
1081 ATTCAGCTATTCTGGAGATCAGCAGCCCTAAGAGATGGGAGTACGACCTGATCTGGTCC 1140
1141 TGGCGCTCCCTAAGCATGTCTCCAGGG 1168

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FIG. 1 Sequence of human *bak*. **a**, Nucleotide sequence and predicted amino-acid sequence of human *bak*. BH1 and BH2 Homology domains are boxed, and the hydrophobic domain is underlined. The nucleotide

b

Bcl-2 (human)	136 ELFRDG-VNW GRVAFPEFGG	155
Bcl-x _L	150 ELFRDG-VNW GRVAFPEFGG	148
Bax (human)	98 DMFSDGNFNW GRVAFYFAS	118
Bak	117 SLFESG-INW GRVALLGFGY	136
Consensus	..F..G..NW GR.VA...F..	
Bcl-2 (human)	187 TWI-QDNGGWDAFVELY	202
Bcl-x _L	180 PWI-QENGWDTFVELY	195
Bax (human)	150 GWI-QDQGWEGLLSYF	165
Bak	170 -WIAQR-GGWVAALNLG	184
Consensus	.WI.Q..GGW.....	

sequence data will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number X84213. **b**, Homology in the BH1 and BH2 domains between human Bak, human Bcl-2, human Bax and human Bcl-x_L. The consensus sequence is also shown. **METHODS.** **a**, The complete E1B 19K coding sequence was cloned in frame with the GAL4-binding domain into pASCYH2, a derivative of pAS1 (ref. 18). This was co-transformed with 5×10^6 cDNAs from an EBV-transformed B-cell library (Clontech) into yeast Y190, a derivative of yeast Y153 (ref. 18). The transformants were plated onto SD medium lacking histidine, tryptophan and leucine in the presence of 25 mM 3-aminotriazole (Sigma). After incubation at 30 °C for 10 days, the colonies were screened for LacZ activity using a freeze-fracture assay¹⁸. Putative interacting clones were back-extracted from yeast²⁷ and rescreened against vector alone to eliminate false positives. Secondary positives were then sequenced in both directions using Sequenase (US Biochemicals). The resulting sequences were used to scan the GenBank and EMBL databases for homologous sequences using the FASTA program. Predicted open reading frames were searched against SwissProt A 350-base-pair XhoI/SalI fragment from the original *bak* clone was used to screen 1×10^6 PFU of a JURKAT cDNA library (Stratagene). All positives were excised *in vivo* according to the manufacturer's instructions. The largest of the clones was sequenced in both directions using Sequenase (US Biochemicals). Structural prediction of the C-terminal hydrophobic domain was performed using the algorithm of Kyte-Doolittle with the GeneWorks 2.3.1 (IntelliGenetics) sequence analysis package for the Macintosh. **b**, Bak protein sequence was aligned against human Bcl-2, Bax and Bcl-x_L using the GeneWorks sequence analysis package.

hours after NGF deprivation, only 10% of *bak*-microinjected neurons survived as opposed to 45–50% of either uninjected neurons or neurons injected with a *bak*-antisense construct. This difference is apparent after 14 hours. These neurons had the characteristic morphology of apoptotic cells, including a pyknotic nucleus (data not shown), suggesting that the death was by apoptosis.

As Bak interacts with E1B 19K in the two-hybrid system, we tested whether *bak* could antagonize the protective effects of E1B 19K. An E1B 19K expression vector was co-injected with an excess of *bak* in the sense or antisense orientation. Whereas the ability of E1B 19K to protect was not affected by antisense *bak*, it was completely blocked by sense *bak* (Fig. 2b). These results suggest that Bak functions in an analogous way to the Bcl-2-binding protein Bax, which acts in opposition to Bcl-2 to accelerate apoptosis²². Like Bax, Bak is expressed in a wide variety of tissues (Fig. 3). Using the yeast two-hybrid system, we tested whether Bak might interact with Bcl-2 or other mem-

bers of the Bcl-2 family (Fig. 4). In this experiment Bak does not appear to dimerize with Bcl-2, Bcl-x_s (ref. 13), Bax, or with itself. Failure to demonstrate homodimerization of Bak in yeast suggests that, unlike Bax, initiation of apoptosis by Bak could depend on interaction with an as-yet unidentified partner. Bak does interact with Bcl-x_L (ref. 17), however, and although the biological significance of this interaction is unknown it is possible that Bak and Bcl-x_L heterodimerize and function in a similar way to Bax/Bcl-2 (ref. 22). As *bcl-x_L* can protect a B-cell line from immunosuppressant-induced apoptosis whereas *bcl-2* cannot²³, these heterodimers may function in a cell-specific manner. Northern blots indicate that *bcl-x_L* has a more restricted tissue distribution than *bak* (data not shown), suggesting that Bak might interact with other Bcl-2-like proteins in tissues where Bcl-x_L is absent.

Our demonstration that overexpression of *bak* can block the protective function of E1B 19K in neurons suggests that the interaction seen in yeast also occurs in higher cells. During the normal course of adenoviral infection, however, we predict that

FIG. 2 Effects of *bak* on neuronal survival. **a**, Kinetics of survival of NGF-deprived neurons overexpressing *bak*. Sympathetic neurons were microinjected with an expression vector for *bak* in either the sense (S, filled circles) or antisense (AS, squares) orientation; open circles represent uninjected neurons. Three hours later, NGF was withdrawn from the culture medium. Survival of neurons was assayed at 6, 14, 18, 24 and 48 h after microinjection. **b**, *bak* antagonizes the survival effects of E1B 19K. Neurons were microinjected with expression vectors for E1B 19K and *bak* in the sense (S) or antisense (AS) orientation. Survival of neurons was assayed two days after NGF deprivation.

METHODS. Sympathetic neurons from cervical superior ganglia of newborn rats were cultured as described²¹. In each experiment, between 100 and 200 neurons were microinjected with each expression vector using experimental procedures described in ref. 21. Three hours post-injection, NGF was withdrawn from the medium, which was supplemented with antibodies to NGF and in which rat serum concentration was reduced from 5 to 2.5%. Estimated survival of neurons was based on morphological criteria and Hoescht stain

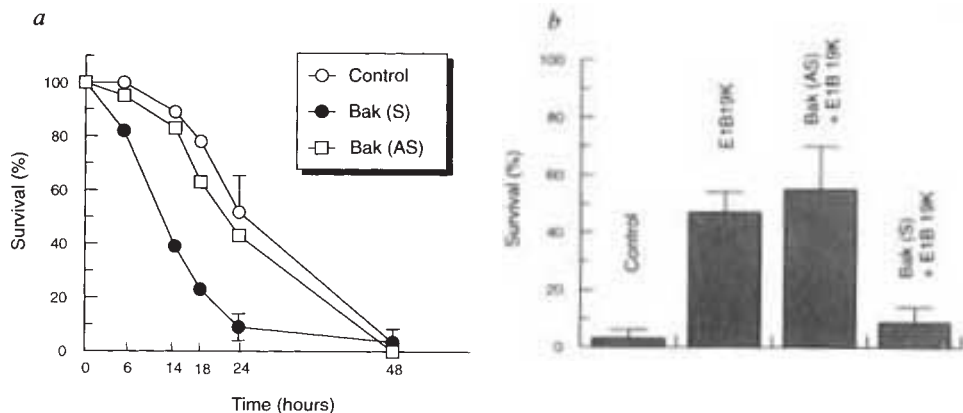


FIG. 3 Northern blot analysis of *bak* expression. **a**, Multiple tissue northern blot (human cell line). Lanes: 1, promyelocytic leukaemia HL-60; 2, HeLa cell S3; 3, chronic myelogenous leukaemia K-562; 4, lymphoblastic leukaemia MOLT-4; 5, Burkitt's lymphoma Raji; 6, colorectal adenocarcinoma SW480; 7, lung carcinoma A549; 8, melanoma G361. **b**, Multiple tissue northern blot (human II). Lanes: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral blood leukocytes.

METHODS. Multiple tissue northern blots (Clontech) were hybridized with a ³²P-labelled 350-base-pair *Sall*/*Xho*I fragment of *bak*. The blot was exposed to Kodak XAR-5 film with an intensifying screen for 9 d at -70 °C. Hybridization to a human β -actin probe is shown as an internal control for loading (3 days exposure with intensifying screen at -70 °C).

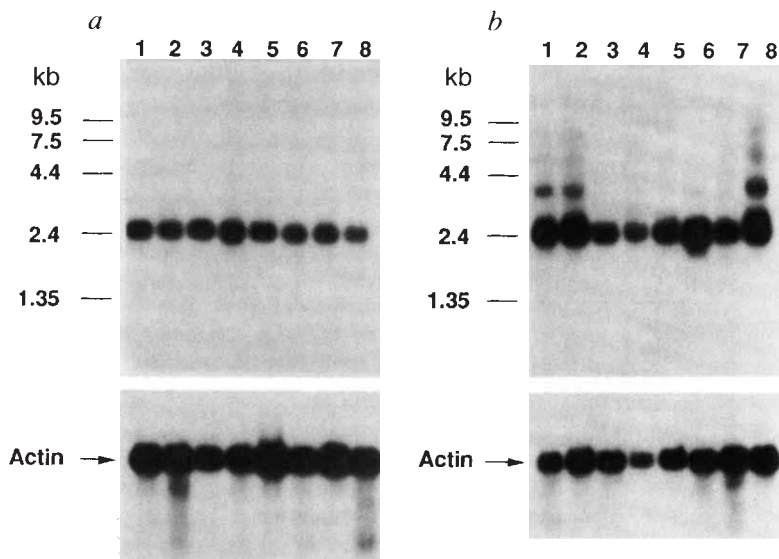
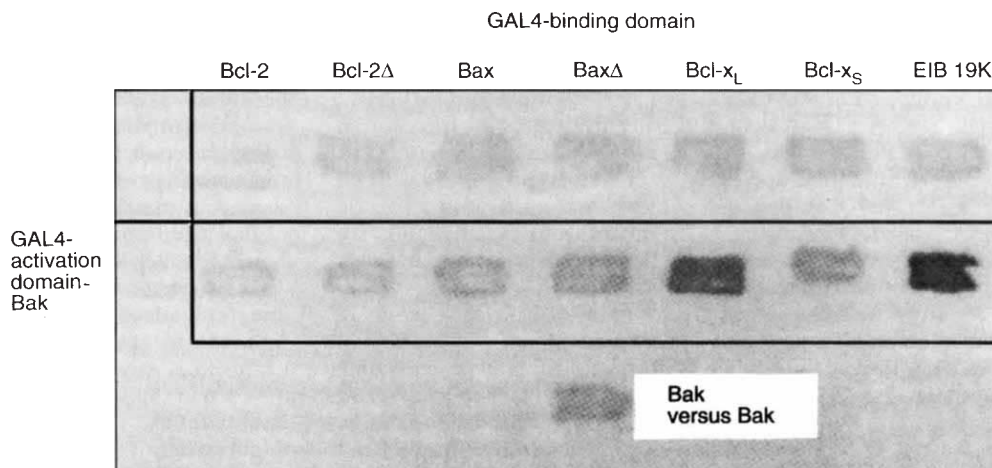


FIG. 4 Interactions between Bak and Bcl-2 family members.

METHODS. Yeast Y190 were transformed with pACT and pASCYH2 vectors¹⁸ expressing the various *bcl-2* family members and *bak* as fusions with the GAL4 activation or binding domains. Bcl-2Δ is a mutant of Bcl-2 lacking the hydrophobic domain. BaxΔ is a mutant of Bax lacking amino acids 164–192. The cells were grown for 2 d at 30 °C on SD medium lacking tryptophan and leucine. Colonies were assayed for LacZ activity using a standard freeze-fracture assay¹⁸. Darkening indicates interaction.



levels of E1B 19K would be sufficient to block the cellular apoptotic response mediated by *bak*. Because transcriptional inhibitors rescue neurons deprived of NGF, we are investigating whether *bak* is transcriptionally upregulated in these cells and, by titrating the amount of *bak* used for microinjection, whether E1B protects neurons in the presence of Bak. The identification of other proteins that bind E1B 19K and the colocalization of nuclear lamina²⁰ indicates that E1B 19K has additional cellular targets, so we are also investigating which of these is essential for E1B 19K inhibition of apoptosis by using E1B 19K mutants that are defective in preventing apoptosis²⁴.

Our results provide new information on the mechanism of action of E1B 19K. This protein does not function by upregulating Bcl-2 (ref. 2), rather Bcl-2 and E1B 19K can each inhibit p53-dependent adenovirus E1A-induced apoptosis^{6,24–26}. On the basis of the modest sequence homology between E1B 19K and Bcl-2, it has been suggested that E1B 19K could be a viral homologue of cellular Bcl-2²⁴. Our results, however, indicate that the E1B 19K protein functions, at least in part, by inhibiting Bak, which normally accelerates apoptosis in response to NGF deprivation. It will be interesting to see whether *bak* helps induce apoptosis in response to stimuli such as anti-Fas and tumour-necrosis factor- α . □

Induction of apoptosis by the Bcl-2 homologue Bak

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CELLS are eliminated in a variety of physiological settings by apoptosis, a genetically encoded process of cellular suicide^{1,2}. Apoptosis comprises an intrinsic cellular defence against tumorigenesis, which, when suppressed, may contribute to the development of malignancies³. The *bcl-2* oncogene, which is activated in follicular lymphomas, functions as a potent suppressor of apoptosis under diverse conditions⁴. Here we describe the complementary DNA cloning and functional analysis of a new Bcl-2 homologue, Bak, which promotes cell death and counteracts the protection from apoptosis provided by Bcl-2. Moreover, enforced expression of Bak induces rapid and extensive apoptosis of serum-deprived fibroblasts. This raises the possibility that Bak is directly involved in activating the cell death machinery.

Cell death is controlled, in part, by a complex interplay between different Bcl-2 family members^{4,5}. To gain a greater understanding of this regulation network, we sought to identify novel genes encoding proteins related to Bcl-2. Amplification by polymerase chain reaction (PCR) and cloning was undertaken using degenerate oligonucleotide primers corresponding to the most conserved domains among known Bcl-2 homologues, referred to as Bcl-2-homology domains I and II⁵. A significant fraction of the clones obtained constituted part of a novel Bcl-2-related gene, designated *bak* (for Bcl-2 homologous antagonist/killer). Northern blot analysis of RNA prepared from a variety of primary human tissues identified a single 2.2-kilobase (kb) *bak* messenger RNA in all tissues examined (not shown). The deduced Bak protein sequence shows highest homology to the human Bcl-2 and Bcl-x_L⁶ proteins, especially in the region encompassing domains I and II (Fig. 1). Bak, like Bcl-2, contains a stretch of hydrophobic residues at its carboxy terminus, ending with several basic residues which, by analogy to Bcl-2, may mediate membrane localization.

The extensive homology between Bak and Bcl-2 suggested that Bak might also regulate apoptosis. To test this possibility, we examined the effect of Bak expression in FL5.12 cells, an

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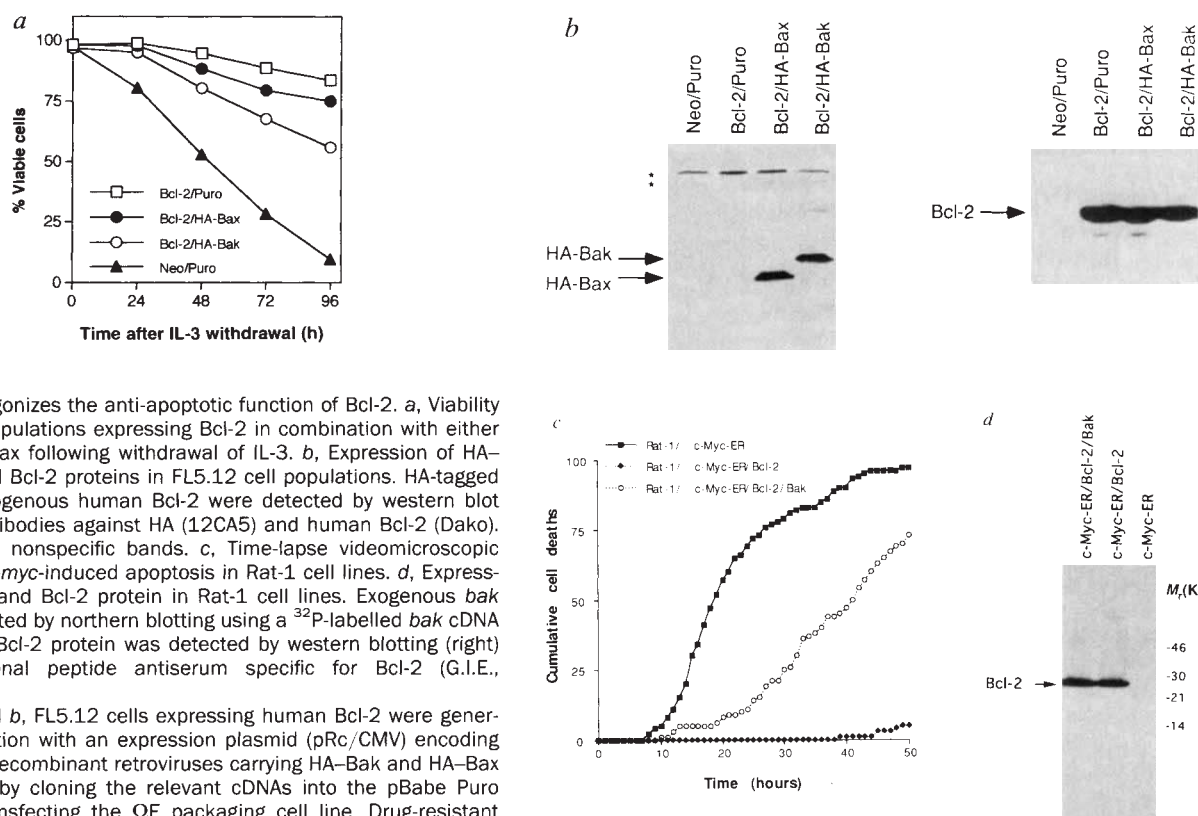


FIG. 3 Bak antagonizes the anti-apoptotic function of Bcl-2. **a**, Viability of FL5.12 cell populations expressing Bcl-2 in combination with either HA-Bak or HA-Bax following withdrawal of IL-3. **b**, Expression of HA-Bak, HA-Bax and Bcl-2 proteins in FL5.12 cell populations. HA-tagged proteins and exogenous human Bcl-2 were detected by western blot analysis with antibodies against HA (12CA5) and human Bcl-2 (Dako). Asterisk denotes nonspecific bands. **c**, Time-lapse videomicroscopic quantitation of *c-myc*-induced apoptosis in Rat-1 cell lines. **d**, Expression of *bak* RNA and Bcl-2 protein in Rat-1 cell lines. Exogenous *bak* mRNA was detected by northern blotting using a ³²P-labelled *bak* cDNA probe (left) and Bcl-2 protein was detected by western blotting (right) using a polyclonal peptide antiserum specific for Bcl-2 (G.I.E., unpublished).

METHODS. **a** and **b**, FL5.12 cells expressing human Bcl-2 were generated by transfection with an expression plasmid (pRc/CMV) encoding human Bcl-2 α . Recombinant retroviruses carrying HA-Bak and HA-Bax were generated by cloning the relevant cDNAs into the pBabe Puro vector²² and transfecting the Ω E packaging cell line. Drug-resistant cells that arose after infection of Bcl-2-expressing FL5.12 cells with retroviruses expressing HA-Bak, HA-Bax, or puromycin alone, were pooled and analysed as cell populations. The neomycin/puromycin population is a G418-resistant FL5.12 cell line that does not express exogenous Bcl-2, infected with the puromycin-encoding retrovirus vector alone. **c** and **d**, Rat-1/c-Myc-ER, Rat-1/c-Myc-ER/Bcl-2 have been described¹¹; Rat-1/c-Myc-ER/Bcl-2/Bak cells were generated by superinfection of these cells with a retrovirus carrying a *bak* cDNA derived

from pBabe Hygro²². After being deprived of serum, c-Myc was activated by addition of β -oestradiol (2 μ M), and cells were observed by time-lapse videomicroscopy as described¹⁰⁻¹². Each field started with 100 cells and images were taken at a rate of 12 frames per hour. Also, four independent clonal isolates of each cell type were monitored for apoptosis by Trypan-blue dye exclusion and gave results consistent with the time-lapse data (not shown).

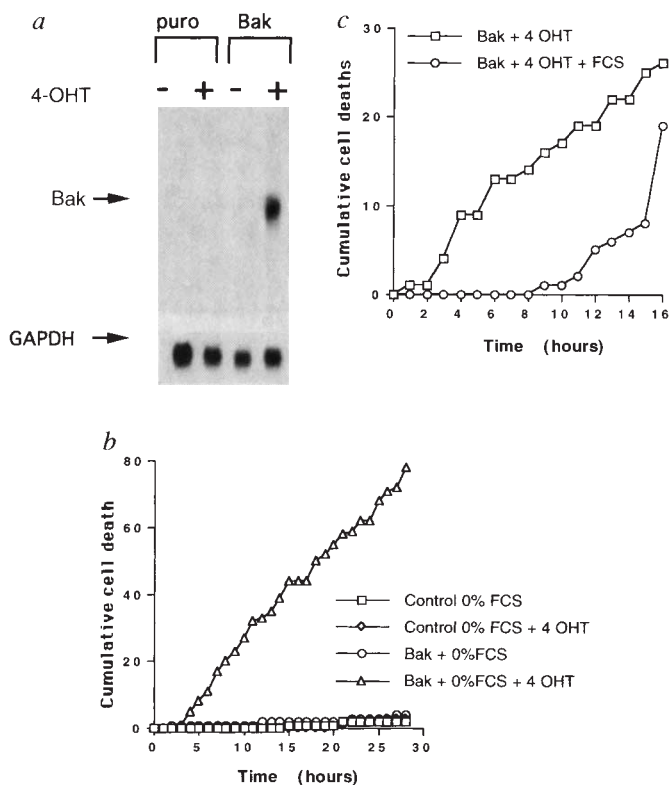


FIG. 4 Induction of apoptosis by Bak in Rat-1 fibroblasts. **a**, Induction of *bak* RNA in Rat-1/Bak cells. RNA (10 μ g) prepared from puromycin-resistant Rat-1 cells and Rat-1/Bak cells two hours after addition of 4-hydroxytamoxifen (4-OHT) to a final concentration of 100 nM, was analysed by northern blotting. RNA from uninduced cells (no 4-OHT) was also analysed. **b**, Time-lapse videomicroscopic analysis of apoptosis in Rat-1 cells following induction of Bak expression. Consistent results were obtained with four independent clones of each cell type when monitoring cell viability by dye exclusion. **c**, Effect of serum in delaying onset of Bak-induced apoptosis. Bak was induced either in the absence or presence of 10% fetal calf serum (FCS) and the matched cultures examined by time-lapse videomicroscopy.

METHODS. A GalER-VP16 fusion¹³ that is regulated by 4-OHT was provided by M. Busslinger. The *bak* cDNA was cloned into a pSP65 derivative, which contains a synthetic promoter containing GAL4 binding sites, that is conditionally transactivated by the GalER-VP16 fusion¹³. Rat-1/GalER-VP16 cells were either co-transfected with pSP65/Bak (10 μ g) and the puromycin-resistance vector J6 Ω puro (1 μ g), or transfected with J6 Ω puro alone. The resulting puromycin-resistant cell lines both express the 4-OHT-regulated GalER-VP16 transactivator; the Rat-1/Bak cells additionally contain *bak* under the control of a GalER-VP16-responsive promoter. Subconfluent Rat-1/puro and Rat-1/Bak cells were deprived of serum for 48 h and Bak expression was activated by addition of 4-OHT. In parallel cultures, 4-OHT was omitted. To examine the effects of serum on Bak-induced apoptosis, identical cell cultures were treated with 4-OHT in either the presence or absence of 10% FCS. Apoptotic cell deaths were recorded as described¹⁰⁻¹².

functions as an oestrogen-activatable transactivator specific for GAL4-responsive promoters¹³. Rat-1 cells expressing GalER-VP16 were transfected with a vector that carries *bak* under the control of a synthetic promoter containing GAL4 DNA-binding sites. Upon addition of the oestrogen receptor agonist 4-hydroxytamoxifen (4-OHT), expression of the *bak* transgene is markedly induced in these cells (Fig. 4a) and results in rapid and extensive cell death in serum-deprived Rat-1 fibroblasts (Fig. 4b). Time-lapse videomicroscopy showed that cell death bore all the morphological features of apoptosis. These results demonstrate that enforced expression of Bak is sufficient to induce apoptosis in serum-deprived fibroblasts. Cell death was significantly delayed when Bak was induced in the presence of 10% serum, demonstrating that serum cytokines can mitigate Bak-triggered apoptosis (Fig. 4c). *c-myc* is one of several other genes, including *c-fos*, *E2F* and *E1a* (refs 14–16) that drive cell proliferation and also induce apoptosis in serum-deprived fibroblasts. In contrast, Bak-induced apoptosis is not accompanied by entry into the cell cycle (not shown) even though it is suppressed by serum.

The similarities between the activities of Bak and Bax place them in the same functional category as Bcl-2-related proteins that accelerate cell death and oppose Bcl-2 function. The short form of Bcl-x (Bcl-x_s) can also abrogate Bcl-2 function, but Bcl-x_s expression by itself does not accelerate cell death upon cytokine withdrawal and, in contrast to Bak and Bax, Bcl-x_s lacks the highly conserved Bcl-2-homology domains I and II (ref. 6). The functional resemblance of Bak to Bax in some assays was unexpected, given that Bak shares more overall sequence homology with Bcl-2 and Bcl-x_L than with Bax. It is at present unclear whether there are structural elements shared by Bak and Bax that are integral to their abilities to promote cell death. The *bak* gene has been independently isolated by two other groups, who also demonstrate that enhanced cell death accompanies its expression^{17,18}.

The molecular mechanism by which Bak induces apoptosis is likely to involve the same evolutionarily conserved cell death regulatory pathway influenced by its relatives, Bcl-2, Bcl-x and Bax. One possibility is that Bak operates solely to mitigate the protective function of relatives like Bcl-2 or Bcl-x_L. By analogy to Bax^{4,9}, this would presumably involve direct molecular interactions with survival proteins. Indeed, we have shown that Bak interacts with both Bcl-2 and (more strongly) Bcl-x_L, but not Bcl-x_s, *in vitro* (not shown). If Bak triggers apoptosis only by antagonizing its anti-apoptotic kin, this implies that serum-deprived Rat-1 fibroblasts are kept alive by virtue of the continuous action of one or more Bcl-2 homologues. Rat-1 fibroblasts do express low levels of both Bcl-2 and Bcl-x_L: they also express Bax, but no detectable Bcl-x_s, A1 (ref. 19) or Mcl-1 (ref. 20) (E.A.H., A. G. Fraser and G.I.E., unpublished data). A possible alternative mechanism by which Bak promotes apoptosis could be that Bak (and perhaps Bax) acts by directly activating the apoptotic pathway or by functioning as components of the cell death machinery. In this case Bcl-2 and Bcl-x_L might suppress apoptosis by inhibiting the death function of Bak and/or Bax, presumably through direct physical interaction. □

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Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak

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MEMBERS of the Bcl-2 family of proteins are characterized by their ability to modulate cell death. Bcl-2 and some of its homologues inhibit apoptosis^{1–4}, whereas other family members, such as Bax, will accelerate apoptosis under certain conditions⁵. Here we describe the identification and characterization of a complementary DNA that encodes a previously unknown Bcl-2 homologue designated Bak. Like Bax, the *bak* gene product primarily enhances apoptotic cell death following an appropriate stimulus. Unlike Bax, however, Bak can inhibit cell death in an Epstein-Barr-virus-transformed cell line. The widespread tissue distribution of Bak messenger RNA, including those containing long-lived, terminally differentiated cell types, suggests that cell-death-inducing activity is broadly distributed, and that tissue-specific modulation of apoptosis is controlled primarily by regulation of molecules that inhibit apoptosis.

The proposal, and subsequent confirmation of apoptotic cell death in the myocardium following ischaemia and reperfusion^{6,8}, led us to search for Bcl-2 homologues that might mediate or otherwise regulate this clinically important process. The Bcl-2 homology-1 and -2 (BH1 and BH2) domains are highly conserved among family members⁹. We designed degenerate oligonucleotides encoding amino-acid sequences within these regions and used them as primers in the polymerase chain reaction (PCR) to clone cDNA fragments encoding Bcl-2 homologues. One new homologous sequence was identified and used to isolate full-length cDNA sequences from human heart and Epstein-Barr (EBV)-transformed human B-cell libraries. An open reading frame within these cDNA clones encodes a novel protein of 211 amino acids (relative molecular mass (*M_r*) 23.4 K). The protein product, Bak (for Bcl-2-homologous antagonist/killer) exhibits marked structural similarity to Bcl-2 and other Bcl-2 family members, particularly in the BH1 and BH2 domains (Fig. 1), where it shares 53% amino-acid sequence identity with Bcl-2. Overall, Bak is 25, 22 and 19% identical to Bcl-2, Bcl-x_L and Bax, respectively. Bak also contains, at its carboxy terminus, a hydrophobic transmembrane domain, indicating that Bak exists as an integral membrane protein.

Southern blot analysis of human genomic DNA and human/rodent somatic cell hybrid DNAs indicated the existence of three closely related *bak* genes. DNA sequence analysis of *bak*-specific PCR products amplified from the somatic cell hybrid DNAs,

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localized the *bak* gene to chromosome 6, a second gene, designated *bak-2*, to chromosome 20, and a third gene, *bak-3*, to chromosome 11. Additional Southern blot analyses suggest that the *bak* gene contains at least three exons and spans ~6 kilobases (kb; result not shown). DNA sequence analysis of cloned *bak-2* and *-3* genes showed that the *bak-2* gene has no introns, and its deduced protein product is 97% identical to Bak. The *bak-3*

gene sequence also lacks introns but clearly represents a processed pseudogene, possessing many hallmarks of such a structure¹⁰.

RNA blot analysis showed that expression of *bak* mRNA was widespread in different tissues as an ~2.4 kb transcript (Fig. 2). In adult tissues, the signals were seen in all tissue analysed, with the highest levels being found in the heart and skeletal muscle



(Fig. 2a, b). In contrast to *Bcl-2*, *bak* mRNA was also found at relatively high levels in the liver, pancreas and kidney. Heart tissue was also found to express the highest level of *bak* mRNA in all fetal tissues analysed (Fig. 2c). Further analysis of individual regions of the adult brain showed that *bak* mRNA was present at relatively high levels in each region (Fig. 2d).

The prolymphocytic FL5.12 cell line has been used extensively for the study of apoptosis sensitivity following interleukin-3 (IL-3) withdrawal^{4,11}. As with *Bax*⁵, stably transfected FL5.12 cells overexpressing *bak* cDNA showed an enhanced sensitivity to IL-3 withdrawal (Fig. 3a). We also investigated the effect of overexpressed Bak in the EBV-transformed WI-L2-729HF₂ (WI-L2) cell line¹², a lymphoblastoid cell line that expresses endogenous Bak and is sensitive to various apoptotic stimuli. In contrast to its activity in the FL5.12 cell line, overexpressed Bak paralleled *Bcl-2* in its activity, and could inhibit apoptosis induced by serum deprivation in transfected WI-L2 cells (Fig. 3b). Consistent with this observation, Bak could also inhibit cell death induced by treatment with the cytotoxic agent menadione. Interestingly, *Bcl-2* was less effective in its protective activity in this cell line (Fig. 3c). In response to each of these treatments, overexpressed *Bax* neither inhibited nor accelerated death of WI-L2 cells (Fig. 3b, c). To verify that these effects were due to Bak

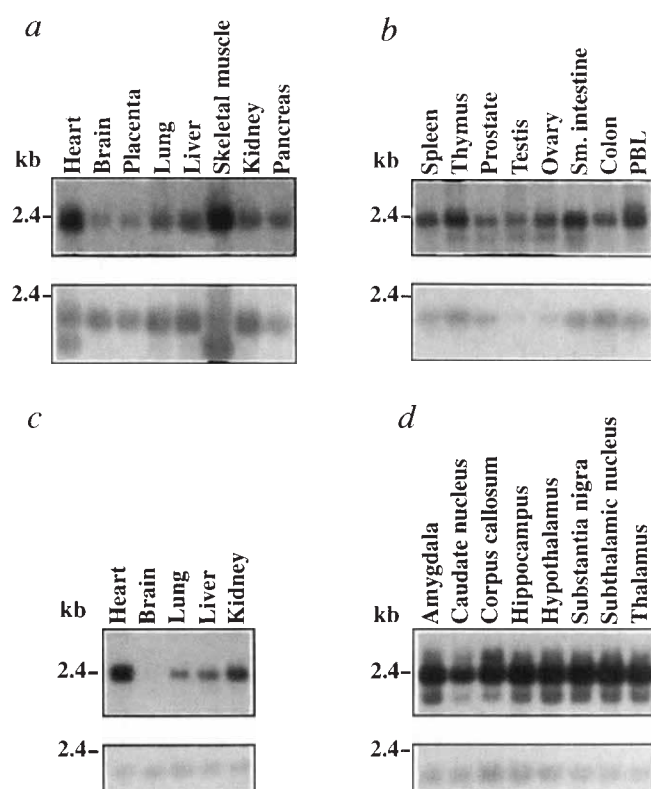
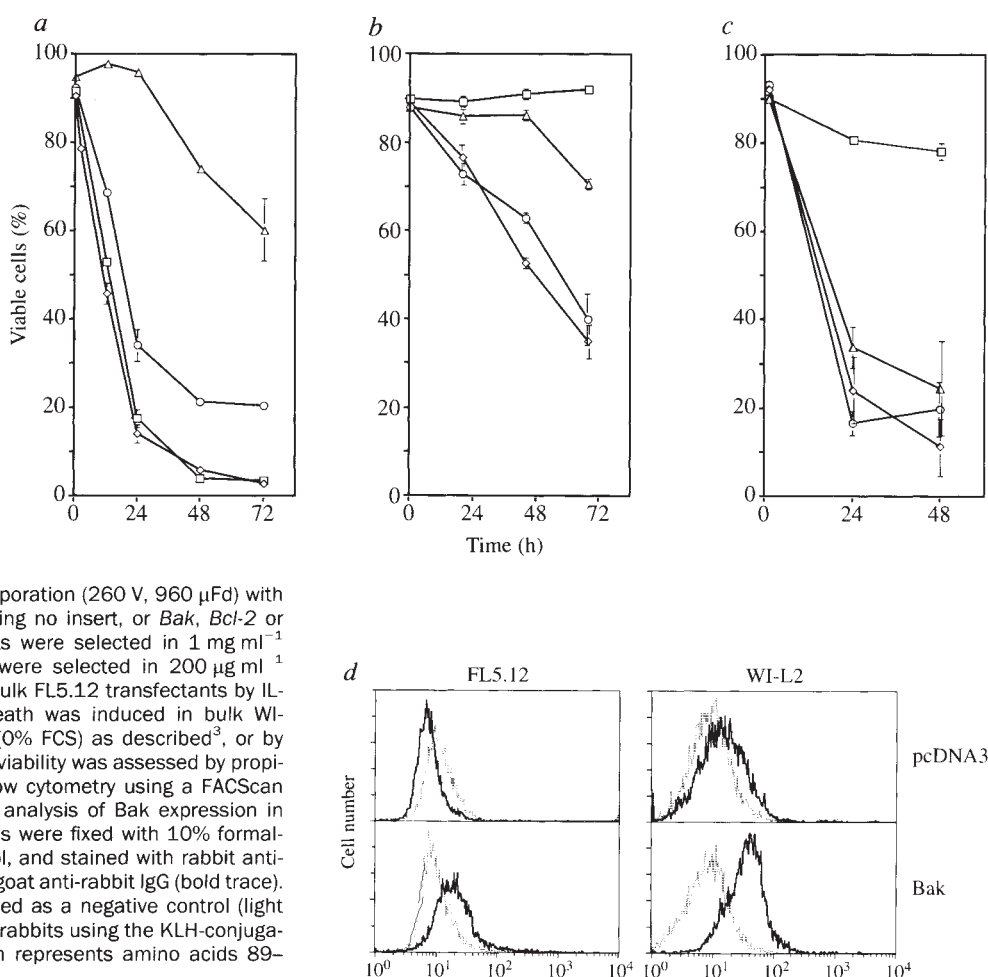


FIG. 2 a–d, Northern blot analysis of human tissue RNAs using *Bak* cDNA probe. Multiple-tissue northern blots (Clontech) containing ~2 µg poly(A)⁺ RNA from adult tissues (a, b), fetal tissues (c), or regions of the brain (d), were hybridized with the *Bak* cDNA probe (top) and washed as for Fig. 1. Blots were also probed with an actin cDNA probe (B-actin, bottom) to verify that comparable levels of poly(A)⁺ mRNAs were present in each lane.

FIG. 3 The effect of Bak expression on cell death. a, Cell viability following IL-3 deprivation of stably transfected FL5.12 cells containing the pcDNA3 vector control (○), *Bak* (□), *Bax* (◇) or *Bcl-2* (△) vectors. Cell viability following b, serum deprivation, or c, menadione treatment of stably transfected WI-L2 cells containing the pcDNA3 control (○), *Bak* (□), *Bax* (◇) or *Bcl-2* (△) vectors. Data are presented as the mean ± s.d. from triplicate samples. Similar results were obtained using at least three independently derived bulk transfectants for each experiment. d, Detection of Bak in transfected cells by immunofluorescence. METHODS. Murine FL5.12 cells were cultured in WEHI-3-conditioned medium¹⁹ supplemented with 40 µM 2-mercaptoethanol. WI-L2 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. Cells were transfected by electroporation (260 V, 960 µF) with the pcDNA3 vector (Invitrogen), containing no insert, or *Bak*, *Bcl-2* or *Bax* cDNAs. Stable FL5.12 transfectants were selected in 1 mg ml⁻¹ G418 and stable WI-L2 transfectants were selected in 200 µg ml⁻¹ hygromycin. Cell death was induced in bulk FL5.12 transfectants by IL-3-deprivation as described^{11,19}. Cell death was induced in bulk WI-L2 transfectants by serum deprivation (0% FCS) as described³, or by treatment with menadione (20 µM). Cell viability was assessed by propidium iodide staining and analysis by flow cytometry using a FACScan (Becton Dickinson). For flow cytometric analysis of Bak expression in transfected WI-L2 and FL5.12 cells, cells were fixed with 10% formaldehyde, permeabilized with 70% ethanol, and stained with rabbit anti-Bak serum, followed by FITC-conjugated goat anti-rabbit IgG (bold trace). Pre-immune serum from rabbits was used as a negative control (light trace). Anti-Bak serum was generated in rabbits using the KLH-conjugated peptide, YDFEQTMLQHLQPT, which represents amino acids 89–103 of Bak.



expression, transfected cells were analysed by flow cytometry using anti-Bak antibodies. Each transfected cell line studied expressed Bak protein that was detectable by this technique (Fig. 3d).

Two cDNAs encoding *Bak* have been isolated independently^{13,14}, one by virtue of the interaction of its protein product with the adenovirus E1B 19K protein¹³. These cDNAs induce apoptosis in primary neurons¹³ and Rat-1 fibroblasts¹⁴, respectively. Bak is thus probably primarily a mediator of cell death which is neutralized by viral anti-apoptotic proteins such as E1B

19K protein. The way in which overexpression of Bak inhibits apoptosis in EBV-transformed cells is unclear. Bak may induce viral anti-apoptotic factors such as the EBV-encoded Bcl-2 homologue BHRF-1 (ref. 3), but its cell-death-inducing activity may also be reversed by its direct heteromeric interaction with such factors. The ubiquitous expression of Bak demands that, at least in long-lived cell types, its activity is controlled by cell-death inhibitory factors. As Bcl-2 expression is quite restricted, the widely expressed Bcl-x_L, or an as-yet unidentified homologue, may serve this function *in vivo*. □

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Molecular basis of antigen mimicry by an anti-idiotope

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IDIOTOPES are antigenic determinants, unique to an antibody or group of antibodies, defined by the reaction of anti-idiotopic antibodies with the antibodies bearing the idiotopes. The ensemble of idiotopes of an antibody constitutes its idiotype. Idiotypes are useful as markers to follow specific antibodies and clones of cells in immune responses and the inheritance of immunoglobulin genes. As external antigens and anti-idiotopic antibodies can competitively bind the combining site of specific antibodies, some anti-idiotopic antibodies may resemble the external antigen, thus mimicking its structure. It has been proposed^{1–5} that an anti-idiotopic antibody, anti-anti-X, may resemble the external antigen X and thus carry its 'internal image', but this idea is not unequivocally supported by the three-dimensional structures of anti-idiotopic antibodies^{6–9}, either because the structures of the external antigen⁸ or of the anti-idiotopic antibody⁷ were unknown, or because the anti-idiotopic antibodies showed no resemblance to the external antigens^{6,9} (reviewed in ref. 10). Functional mimicry of ligands of biological receptors by anti-idiotopic antibodies has been described in several systems (reviewed in ref. 11). But how closely can antibodies mimic antigens at the molecular level? Here we present the crystal structure of an idiotope–anti-idiotope complex between the Fv fragments of the anti-lysozyme antibody D1.3 and the anti-D1.3 antibody E5.2. D1.3 contacts the antigen, lysozyme and the anti-idiotopic E5.2 through essentially the same combining-site

residues. In addition, E5.2 interacts with D1.3, making contacts similar to those between lysozyme and D1.3. Thus, the anti-idiotopic antibody E5.2 mimics lysozyme in its binding interactions with D1.3. Validating these observations, E5.2, used as an immunogen, induces an anti-lysozyme response.

We determined the crystal structure of the idiotope–anti-idiotope complex between recombinant Fv fragments from the monoclonal antibodies D1.3 and E5.2, to a nominal resolution of 1.9 Å. (Fv fragments consist of the variable parts, V_L and V_H, of the light (L) and the heavy (H) chains of the parent antibodies and contain the antibody combining sites.) The complex is formed through contacts from all six complementarity-determining regions (CDR) of each Fv, although the V_H of D1.3 and E5.2 are predominant in such contacts (Fig. 1). The solvent-excluded area of the interface is 1,886 Å² (912 Å² from D1.3, 974 Å² from E5.2, calculated with a water molecule of radius 1.7 Å). The E5.2 residues in contact with D1.3 are: V_L Tyr 32, Tyr 49, Arg 53, Asn 92, Thr 93; V_H Lys 30, His 33, Asp 52, Ala 53, Asn 54, Gln 58, Ile 97, Tyr 98, Tyr 99, Gln 100, Gly 100a, Arg 100b (numbers as in ref. 12). The V_H CDR3 of E5.2 accounts for 77% of the total contacts to D1.3. With the exception of V_L Tyr 49 of both antibodies, no 'framework' residues are involved in interface contacts. However, V_L Tyr 49 of D1.3 makes contacts with the antigen, hen egg lysozyme (HEL), and is thus part of its combining site. This is in contrast with a Fab–Fab complex between D1.3 and another anti-idiotopic antibody, E225 (Fig. 1), in which several framework residues participate in idiotope–anti-idiotope contacts but in which no mimicry of lysozyme can be detected⁶. Also, the affinity of E5.2 for D1.3 (4.3×10^8 M⁻¹ (ref. 13) or 1.4×10^9 M⁻¹, as redetermined here), is closer to that of HEL for D1.3 (2.7×10^8 M⁻¹) than is the affinity of E225 for D1.3 (2.0×10^5 M⁻¹). The lower affinity of E225 for D1.3 has been interpreted¹³ as reflecting the conformational changes that take place in the combining site of D1.3 complexed to E225 (Fig. 1). By contrast, the D1.3 combining site is conformationally similar in its complexes with HEL and E5.2 (Fig. 1; the r.m.s. deviation of positions of all atoms of the thirteen D1.3 residues contacting both HEL and E5.2 (see below) is 0.56 Å).

Of the 18 D1.3 residues that contact E5.2 and the 17 that contact HEL, 13 are in contact with both E5.2 and HEL (Fig. 1a, b). These 13 residues of D1.3 make up 75% (687 Å²) of the

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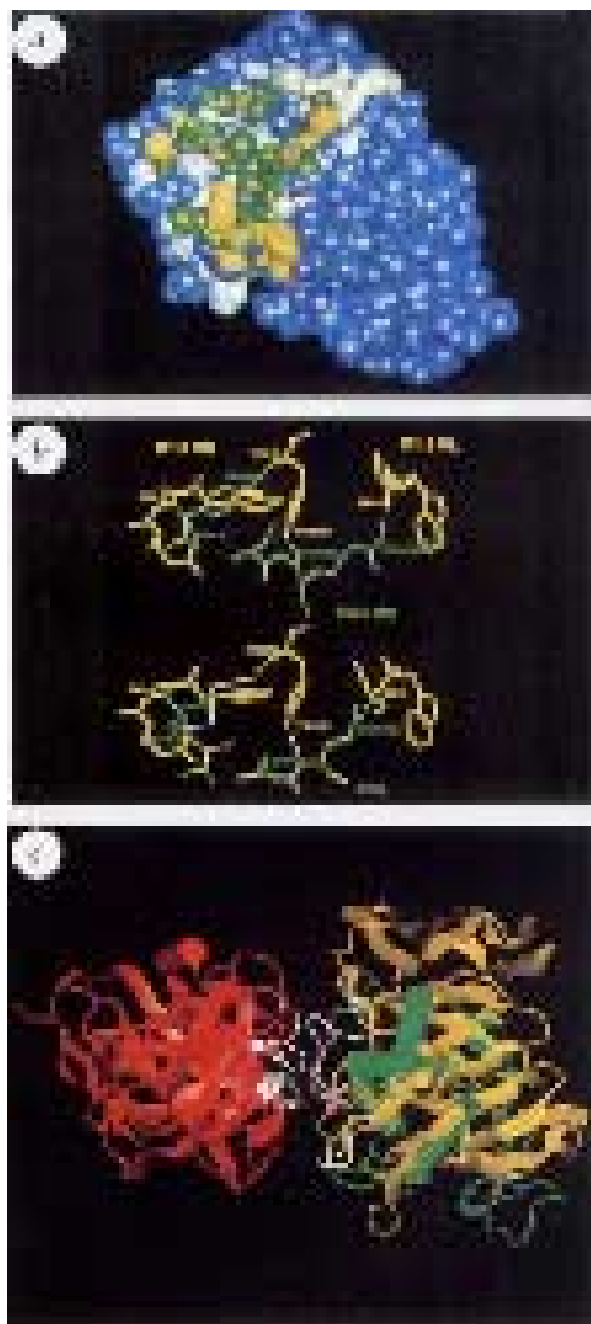
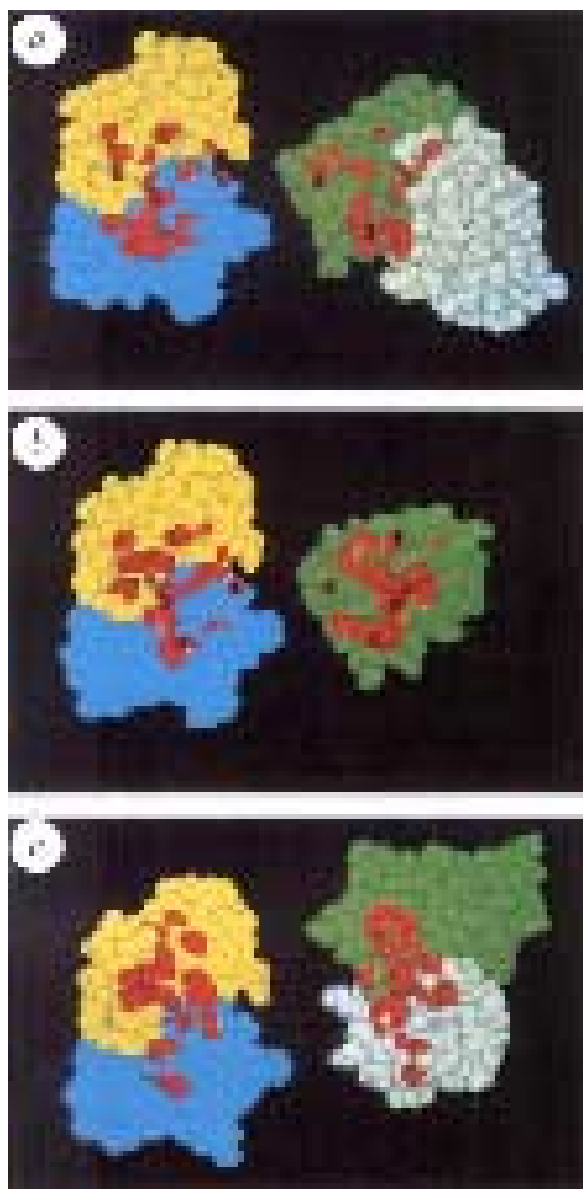


FIG. 1 Comparison of idiotope-anti-idiotope and antigen-antibody interactions. *a*, Contacting atoms (in red) in the D1.3-E5.2 complex. VL D1.3 is yellow, VH blue, VL E5.2 light green, VH green. Residues of D1.3 that contact E5.2 are: VL His 30, Tyr 32, Tyr 49, Tyr 50, Trp 92 and VH Thr 30, Gly 31, Tyr 32, Gly 33, Trp 52, Gly 53, Asp 54, Asn 56, Asp 58, Glu 98, Arg 99, Asp 100, Tyr 101; numbers 1-5 correspond to atoms in Table 1. *b*, D1.3 atoms involved in contacts with HEL (compare with *a*). Residues of D1.3 that contact HEL¹⁴ in VL are: His 30, Tyr 32, Tyr 49, Tyr 50, Thr 53, Phe 91, Trp 92, Ser 93; and in VH: Gly 31, Tyr 32, Trp 52, Gly 53, Asp 54, Arg 99, Asp 100, Tyr 101, Arg 102. Lysozyme is shown in green; atoms numbered 1-5 are listed in Table 1. *c*, Contacting atoms of D1.3 (left) and the anti-idiotope E225 (right). In this idiotope-anti-idiotope complex⁶ the D1.3 side chains (VL, Tyr 50, Trp 92 and VH, Asp 100) have changed conformations to give a very different combining-site structure from that shown in *a* and *b*. In E225, VL is light green, VH is green, and contacting atoms are red.

FIG. 2 Comparison of contacts made by E5.2 and HEL with D1.3 to illustrate the degree of resemblance of anti-idiotope to antigen. *a*, Superposition of E5.2 (blue, with atoms contacting D1.3 in yellow) with HEL (white, with atoms contacting D1.3 in green). The D1.3 α C atoms in the two complexes were superimposed by least squares (r.m.s. deviation, 0.38 Å), giving the superposition of E5.2 and HEL. Numbers 1-5 are as in Fig. 1 and Table 1; atom number 5 of E5.2 is hidden by HEL atom number 5. *b*, Hydrogen bond between E5.2 VH Tyr 98 OH and

D1.3 VH Tyr 101 N superimposes in space with that made between a water molecule and D1.3 VH Tyr 101 N in the D1.3-HEL complex (number 6 in Table 1). Other equivalent hydrogen bonds (listed as 2, 3 and 5 in Table 1) can be seen. *c*, Ribbon diagram of the superimposed structures of lysozyme (green) and FvE5.2 (yellow) in the orientation assumed in their complexes with D1.3 (red). The HEL epitope is shown in magenta, the CDR of E5.2 and D1.3 in white; VH CDR3 of E5.2 closely follows the structure of lysozyme residues 116-121.

METHODS. Crystals belong to space group C2 with $a=152.8$ Å, $b=79.4$ Å, $c=51.5$ Å, $\beta=100.2^\circ$ (ref. 15). X-ray intensities were measured on an R-Axis image-plate detector: 105,969 observations to 1.79 Å resolution were merged to give 41,404 unique reflections with $R_{\text{merge}}=8.5\%$. For reflections with $F>2\sigma(F)$, the data set is 70% complete in the range 7-1.9 Å and 27% complete in the range 2.0-1.9 Å. The structure was solved by molecular replacement with the program AMoRE¹⁶ using FvD1.3 (ref. 14) as a search model. The sequences of FvE5.2 (ref. 15) and FvD1.3 (ref. 17) were fitted to electron density maps. Refinement of atomic coordinates with X-PLOR (ref. 18) gave R -factor = 0.194 for 32,539 reflections with $F>2\sigma(F)$ in the range 7-1.9 Å; r.m.s. deviations from ideal bond lengths and bond angles are 0.016 Å and 2.0° . The model includes 157 water molecules and 3 zinc ions (from the crystallization solution). Further details will be published elsewhere.

TABLE 1 Hydrogen bonds between D1.3 and E5.2, six of which are superimposable in space with those made between D1.3 and its antigen HEL

D1.3 atom	E5.2 atom	Superimposable HEL atom
V _L Tyr 49 OH	V _H Asn 54 Nδ2 (2.8 Å)	—
V _H Thr 30 O	V _H Tyr 98 N (2.9 Å)	—
V _H Asp 54 Oδ1	V _L Tyr 49 OH (2.6 Å)	—
V _H Asn 56 Nδ2	V _L Gln 100 Oε1 (3.2 Å)	—
V _H Asp 58 Oδ1	V _L Gln 100 Nε2 (3.2 Å)	—
V _H Glu 98 Oε1	V _H Tyr 98 OH (2.5 Å)	—
(1) V _L Tyr 50 OH	V _H Gln 58 Nε2 (3.3 Å)	Asp 18 Oδ2 (2.7 Å)
(2) V _L Trp 92 O*	V _H Arg 100b Nη2 (2.9 Å)	—
(2) V _L Ser 93 N*	—	Gln 121 Oε1 (2.9 Å)
(3) V _H Gly 53 N	V _H Tyr 98 O (3.0 Å)	Gly 117 O (2.8 Å)
(4) V _H Arg 99 NH1	V _H Lys 30 O (3.3 Å)	Gly 102 O (2.7 Å)
(5) V _H Tyr 101 OH	V _H Gly 100a O (2.8 Å)	Gln 121 N (3.0 Å)
(6) V _H Tyr 101 N	V _H Tyr 98 OH (3.1 Å)	H ₂ O 749 (3.0 Å)

Numbers (1)–(5) correspond to those in Figs 1 and 2; hydrogen bonds 2, 3, 5 and 6 are shown in Fig. 2b. Distances in Å are hydrogen-bond lengths.

* These hydrogen bonds are superimposable in space because of the ~160° difference, in the two complexes, in the peptide plane conformation of V_L D1.3 at positions 92–93 (see text).

total contacting area with E5.2 and 87% (675 Å²) of that with HEL. Further, the positions of the atoms by which E5.2 contacts D1.3 are close to those of lysozyme that contact D1.3 (Fig. 2). Thus in this respect E5.2 mimics HEL. This mimicry extends to the hydrogen bonding: six of the twelve interface hydrogen bonds in the D1.3–E5.2 complex are structurally equivalent (that is superimposable in space) to hydrogen bonds in the D1.3–HEL interface (Fig. 2b; Table 1), in contrast with the D1.3–E225 complex⁶. The FvD1.3 peptide group at V_L positions 92–93 undergoes a 160° rotation relative to its conformation in the complex with HEL. This allows formation of a hydrogen bond between V_L D1.3 Trp 92 and V_H E5.2 Arg 100b which is spatially close to that made between V_L D1.3 Ser 93 and HEL Gln 121 (Table 1).

There are many water molecules in and around the interface. The positions of 11 of these are conserved within 1 Å in both the D1.3–E5.2 and the D1.3–HEL interfaces. In addition, V_H Tyr 98 of E5.2 protrudes into a pocket of D1.3, whereas in the D1.3–HEL complex water molecules occupy this position, providing very similar contacts (Fig. 2b; Table 1). Thus, solvation contributes to this binding mimicry.

The structural mimicry described, comprising van der Waals contacts, hydrogen bonds and solvent interactions, suggested that E5.2 as an immunogen could provide an 'internal image' of HEL. Accordingly, BALB/c and C57BL mice were immunized with E5.2. E5.2 should be much less effective in inducing an anti-HEL response than polyclonal, xenogeneic preparations of anti-idiotypic antibodies, which would be more immunogenic and diverse and thus more likely to bear an internal image of the antigen. However, the results (Fig. 3a) showed that some mice produced an anti-E5.2 response in which anti-HEL antibodies could be detected. Figure 3b shows that the adsorption by the anti-D1.3 FabE225 has no appreciable effect on the anti-HEL activity of an anti-anti-idiotypic serum induced by immunization with E5.2, suggesting that the induced antibodies are not strictly D1.3-like, in other words that E5.2 did not simply stimulate clones expressing D1.3. Figure 3c indicates that in the serum of a responder mouse the antibodies with anti-HEL activity constitute a small fraction of the total anti-E5.2 antibodies, as expected.

The structure of the idiotope–anti-idiotope complex described here helps us to visualize how antibody CDRs, which are not helical (Fig. 2c), can 'mimic' the structure of an antigen when, as in this case, the epitope is partly α -helical. The fidelity of molecular mimicking will evidently depend on the structure of the antigen or the epitope, the choice of antibody and of anti-idiotypic antibody, plus numerous other complexities of immune responses. In this or in other antigen–antibody systems, a closer

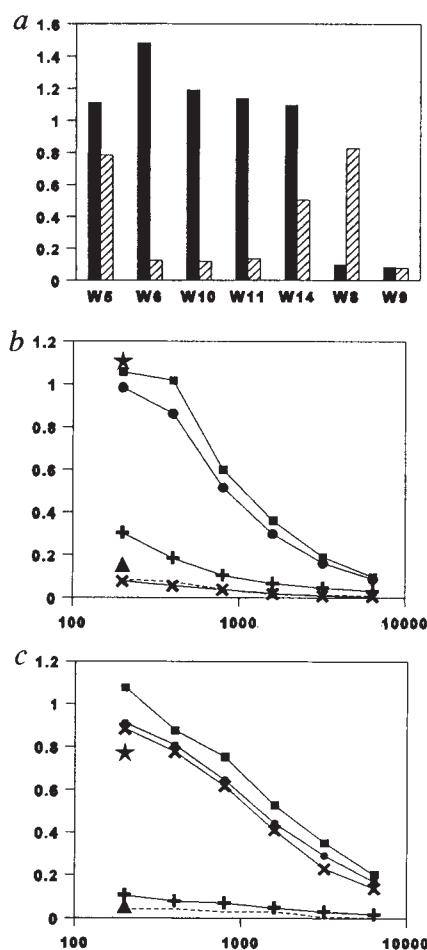


FIG. 3 Immunochemical analysis of E5.2 as a mimic of lysozyme. a, Reactivity of sera from BALB/c (W5 and W6) and C57BL (W10, W11, W14) mice immunized with E5.2, HEL (BALB/c W8) or from control unimmunized mice (BALB/c W9). Sera were tested by enzyme-linked immunosorbent assay (ELISA) against FvE5.2 (black) and HEL (stippled) at 1/200 dilution, except those of W8 (against HEL) and 1/12,000 dilution. Ordinate: optical absorption at 495 nm. (Control unimmunized and HEL-immunized C57BL mice (not shown) were similar to BALB/c controls.) b, Reactivity of W5 anti-E5.2 serum against HEL by ELISA: non-adsorbed (■) and adsorbed with: mAb E225 (●), E5.2 (+) and HEL (x). Controls: serum from W9 (dashed line), unadsorbed D1.3 (★) and E225-adsorbed D1.3 (▲) assayed against HEL. Ordinate, as in a; abscissa, inverse dilutions in log scale. c, Assays as in b but using FvE5.2 as adsorbed antigen.

METHODS. mAb D1.3, anti-idiotypic mAbs E225 and E5.2 and their Fvs were prepared as described^{15,17,19,20}. Male mice (6–7 weeks old) were immunized intraperitoneally with E5.2 coupled to keyhole limpet haemocyanin, first in complete Freund's adjuvant and subsequently (3 times) in incomplete Freund's adjuvant at 20-day intervals. Control mice were immunized with HEL in complete Freund's adjuvant and three weeks later in incomplete Freund's adjuvant. Sera were obtained by orbital bleeding. Duplicate ELISAs were done as described²¹ on plastic plates derivatized overnight with 100 µl of a 20 µg ml⁻¹ solution of FvE5.2 or a 50 µg ml⁻¹ solution of HEL. For adsorptions, sera diluted 1/100 were incubated for 1 h at 25 °C with CNBr-activated 4B-Sepharose (Pharmacia) coupled to 2 mg FabE225, IgGE5.2 or HEL, and the supernatants used for ELISA.

molecular mimicry than that shown in Figs 1 and 2 may be achieved. But these and other results⁵ suggest that the mimicking is functional, involving similar binding interactions (Table 1) rather than exact topological replicas, which in most cases would be almost impossible to achieve, especially for anti-idiotypic

antibodies that 'mimic' molecules that are not proteins. Furthermore, it follows that antigenic mimicry does not depend on amino-acid sequence homology between protein antigens and anti-idiotopic antibodies. □

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High-resolution structure of a DNA helix forming (C·G)*G base triplets

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TRIPLE helices result from interaction between single- and double-stranded nucleic acids. Their formation is a possible mechanism for recombination of homologous gene sequences in nature and provides, *inter alia*, a basis for artificial control of gene activity. Triple-helix motifs have been extensively studied by a variety of techniques, but few high-resolution structural data are available. The only triplet structures characterized so far by X-ray diffraction were in protein–DNA complexes^{1,2} studied at about 3 Å resolution. We report here the X-ray analysis of a DNA nonamer, d(GCGAATTCG), to a resolution of 2.05 Å, in which the extended crystal structure contains (C·G)*G triplets as a fragment of triple helix. The guanine-containing chains are in a parallel orientation. This arrangement is a necessary feature of models for homologous recombination which results ultimately in replacement of one length of DNA by another of similar sequence. The present structure agrees with many published predictions of triplex organization, and provides an accurate representation of an element that allows sequence-specific association between single- and double-stranded nucleic acids.

The nonamer d(GCGAATTCG) crystallizes in space group *P2₁2₁2₁* with one duplex per asymmetric unit (Fig. 1). The duplex consists of an eight-base-pair classical B-form DNA double helix with unpaired guanine bases at its ends. The most striking feature of the structure is the formation of (C·G)*G base triplets as a result of interaction between adjacent duplexes. Each overhanging G residue forms hydrogen bonds with the terminal base pair of the neighbouring double helix, as represented schematically in Fig. 2a.

Two hydrogen-bonding arrangements proposed for (C·G)*G base triplets are shown schematically in Fig. 2b and c. The first of these involves a Hoogsteen hydrogen-bond pattern with a parallel orientation of the third strand with respect to the purine Watson–Crick strand. The second has a reverse-Hoogsteen hydrogen-bond pattern and antiparallel orientation. Molecular modelling studies³ have suggested that the Hoogsteen configuration is energetically preferred for ((C·G)*G)_n homopolymeric triple helices where the third strand has C2'-endo sugars and an *anti* conformation about the glycosidic bond.

The Hoogsteen hydrogen-bond pattern was found in the present structure: see Fig. 3a, where one of the triplets is superimposed on the 2.05 Å electron-density map. Geometrical details for the two (C·G)*G base triplets are given in Fig. 3b. Although crystallographically independent, the two triplets are geometrically very similar. In each, the C·G base pair is linked by normal Watson–Crick hydrogen bonds ranging from 2.70 Å to 2.88 Å. The second guanine base interacts in the major groove with both C and G of the Watson–Crick pair and forms three hydrogen bonds of similar length, between 2.70 Å and 2.94 Å. This central positioning of the third strand base makes strand switching of the third strand unnecessary when binding with duplexes containing both purines and pyrimidines.

The parallel orientation of the sugar–phosphate backbones involving the G bases can be clearly seen in Fig. 3b. In most depictions of (C·G)*G triplets in the Hoogsteen parallel arrangement the possible short N⁴–H...O⁶ contact is not shown as a hydrogen bond^{3,4} because the angles N⁴–H...O⁶ are not linear, or the third base is not centrally positioned with respect to the Watson–Crick base pairs. Although in the present structure the NH...O bond angle is 109–111°, such angles are not uncommonly observed in NH...O hydrogen bonds in crystal structures analysed to high resolution⁵.

In both triplets the three bases are almost coplanar. The angle between base planes is in the range 2.0–13.5° only. The orientation of the three bases corresponds to the sharp energy minimum calculated by Zhurkin⁶.

It has been suggested that triplex formation is associated with partial opening of the Watson–Crick pairs in the direction of the major groove⁷. However, there is no evidence for such an effect in the present structure, where interatomic N...O contacts at both the major and minor groove sides of the pair are equivalent and the opening angles σ (ref. 8) are 5.6° and 5.2°, similar to the corresponding values of 3.8° and 5.5° in the parent dodecamer⁹. This contrasts with the CAP–DNA complex¹, where the hydrogen bonding scheme of the (C·G)*G triplet is comparable to the present structure but the N...O contacts are 3.44 Å and 2.66 Å. However, the triplet bases in the complex are far from coplanar and the distortion, which is also considerable in other parts of the double helix, may well be due to protein binding.

Figure 1 shows the packing of the B-form DNA nonamer in the extended crystal structure. The duplex portions are oriented along the *c*-axis and the adjacent helices interact, in common with several other published examples, by end-to-end stacking. The lateral interactions, however, are novel. Nevertheless, the central AATT part of the present structure is very similar to that in the parent dodecamer⁹, suggesting that the narrowing of the minor groove in this region is related to the sequence rather than to packing effects.

Like all oligonucleotides studied by X-ray diffraction

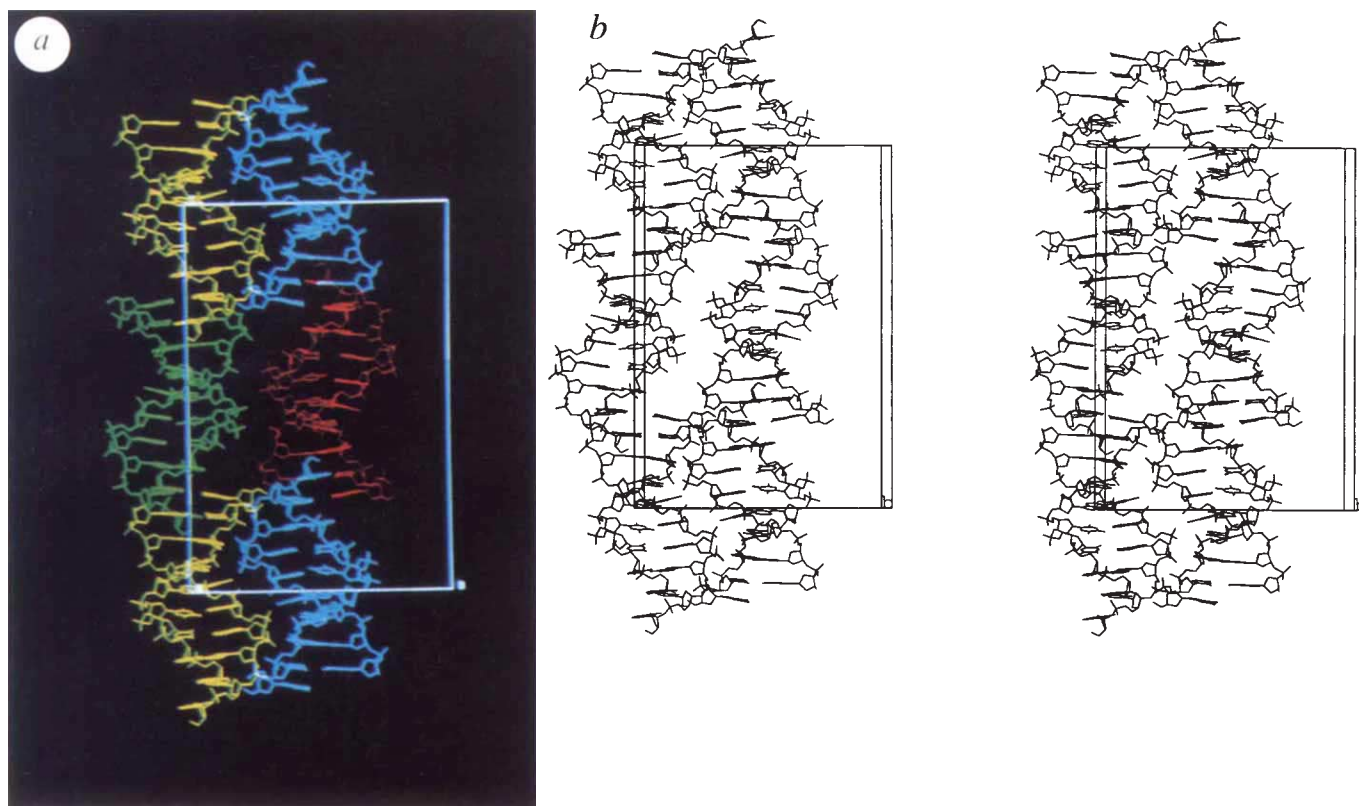


FIG. 1 *a*, Packing diagram of the structure of the nonamer d(GCGAATTCG) showing one layer of molecules as viewed down the crystallographic *a*-axis. *b*, Stereo drawing of the same view. METHODS. The nonamer was synthesized by solid-phase phosphoramidite methods and crystallized from a buffered 0.5-mM solution of

DNA (pH 6.0) containing 54 mM MgCl_2 , 2.4 mM spermine, 11% 2-methyl-2, 4-pentenediol (MPD) equilibrated against 50% MPD by the vapour-diffusion technique at 16 °C. The space group is $P2_12_12_1$ with $a = 22.238$ (4), $b = 37.010$ (2), $c = 54.100$ (2) Å. The asymmetric unit consists of one duplex aligned along the *c*-axis.

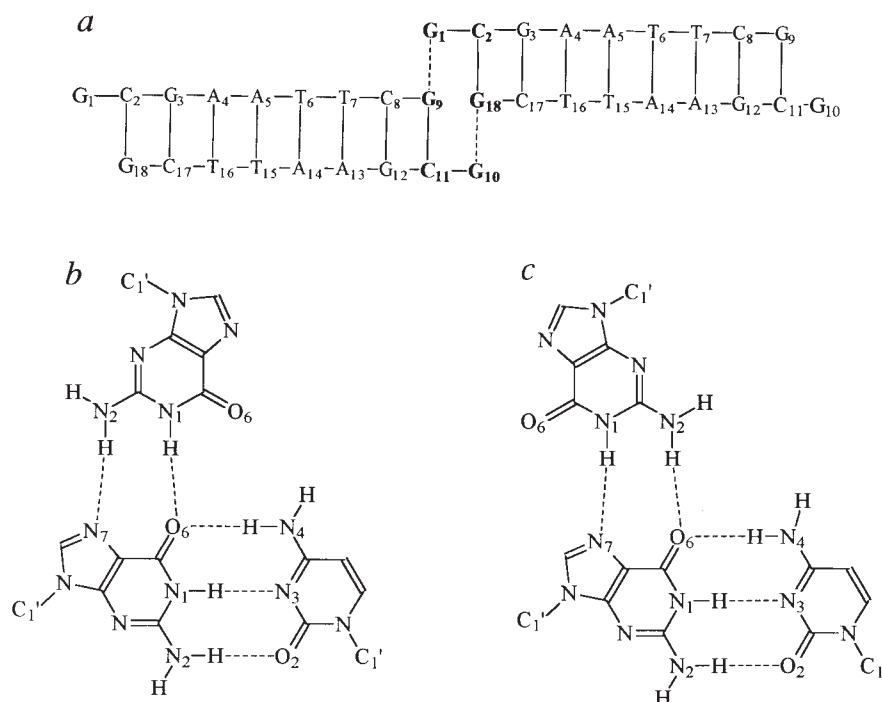


FIG. 2 *a*, Schematic representation of $(C \cdot G)^*G$ triplet formed by overlapping duplexes. The triplets $(C_{11} \cdot G_9)^*G_1$ and $(C_2 \cdot G_{18})^*G_{10}$ are not related by any crystallographic symmetry. *b* and *c*, Alternative hydrogen-bond arrangements in $(C \cdot G)^*G$ triplets: *b*, Hoogsteen; *c*, Reverse Hoogsteen.

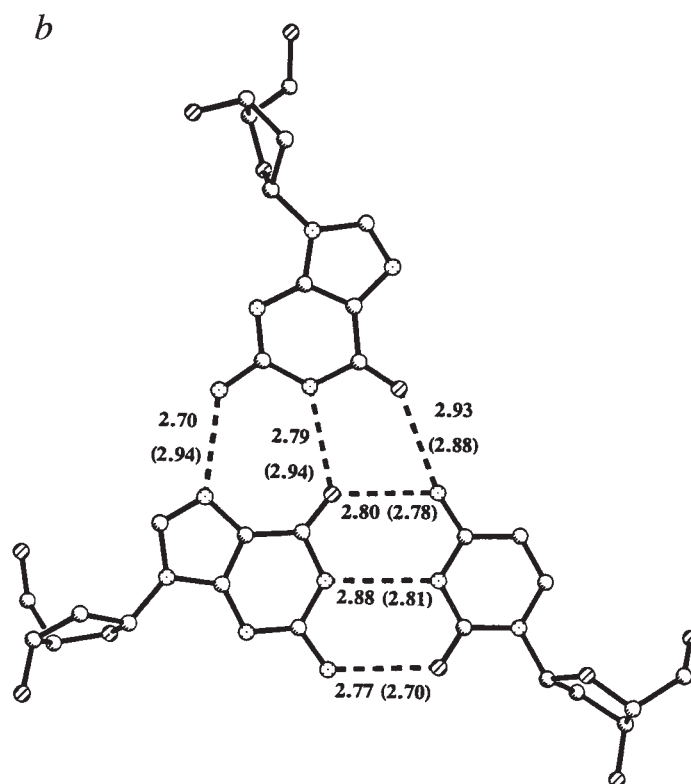
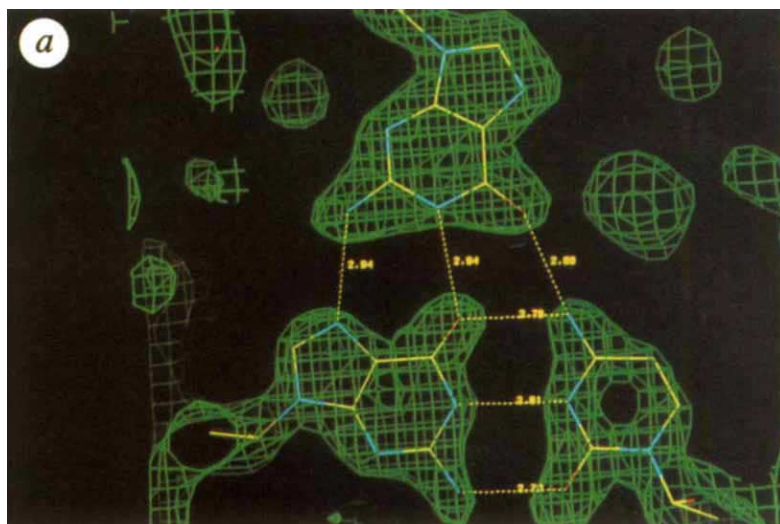
techniques, this structure is also heavily hydrated: 88 water molecules and 2 Mg^{2+} ions were located. One of the Mg^{2+} ions is found in the region of the triplets linking the two together. The mutual orientation of the triplets is further stabilized by a chain of water molecules. The minor groove contains the spine

of hydration, as observed in the B-DNA dodecamer⁹, and the presence of a triplet does not cause a significant perturbation of the duplex.

Interactions between molecules in the solid state can give information about recognition features essential to more com-

FIG. 3 *a*, 2Fo-Fc map showing one of the (C · G)*G triplets. *b*, The (C11 · G9)*G1 triplet as observed in the crystal structure of the nonamer d(GCGAATTCG). Hydrogen-bond distances are in Ångströms, those for (C2 · G18)*G10 are given in parentheses.

METHODS. The X-ray intensities to 2.05 Å from a single crystal (0.31 × 0.15 × 0.13 mm) were collected with a MarResearch Imaging Plate detector using a copper rotating anode source at 18 °C. In total, 22,402 reflections reduced to 2,824 unique reflections with $R_{\text{sym}} = 3.6\%$, and 96% of the reflections have $I > 3\sigma(I)$ (completeness 87%). The structure was solved via the molecular replacement technique, using the program AMoRe (ref. 10). A B-DNA nonamer based on the B-DNA dodecamer⁹ d(CGCGAATTCGCG) was used as a search model. Initial rigid-body refinement led to an R -factor of 43% for the 8–2 Å data. Further refinement using X-PLOR (ref. 11) reduced the R -factor to 16.8% for the 2,379 reflections ($F > 4\sigma(F)$) between 8 Å and 2.05 Å, including 88 water molecules and 2 Mg^{2+} ions. The bond lengths and angles have r.m.s. deviations of 0.015 Å and 3.21°, respectively, from standard values. Final coordinates will be sent to the Brookhaven Protein Data Bank¹². Full details of the X-ray analysis will be published elsewhere.



plex biological structures. Structural concepts for A · U and G · C base pairs still owe much to early crystallization experiments on simple base analogues. Although the present structure characterizes only a single repeat unit of a triple helix, it provides accurate information for use in further experimental and theoretical studies. Attempts to obtain crystals of triple helices from mixtures of appropriate single strands have always yielded fibre patterns. The ability of overhanging bases to form triplexes

opens up possibilities for obtaining ordered crystals of triple helical fragments by extending the length of the overhanging strand and applying crystal-engineering techniques. The information about the precise dimensions of the (C · G)*G triple helical unit and the presence of a hydrogen bond between both the C and G Watson-Crick pair and the third base will need to be taken into account in future theoretical and experimental studies. □

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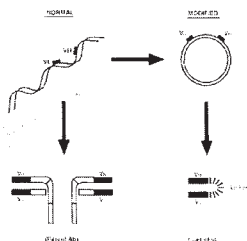
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Concentrating on immunochemistry

Here are some undiluted solutions to your immunology needs — an anti-fluorescein single-chain antibody, monoclonal antibodies to Stat proteins 1–6 and a variety of microplate technologies.

CYTImmune Sciences has added Cytokit Red VEGF to its line of **enzyme immunoassay kits for cytokine detection** (*Reader Service No. 100*). This kit is a polyclonal-based competitive cytokine enzyme immunoassay that measures total cytokines in normal serum in the nanometre per millilitre range. This non-isotopic assay format is designed to allow serum or plasma samples to be measured directly without any pre-assay dilution or preparation, and to negate any interference by blood-borne binding proteins, such as soluble receptors, autoantibodies, or high molecular weight proteins. A full line of Cytokit Red detection kits are available for the detection of human IL-1 α , β , 2–4, 6–8, 10, 13, IFN α , TNF α , and MIP-1 α ; and murine IL-1 α , β , IL-3, IL-6, GM-CSF, and TNF α . Hormone immuno-assay kits are also available for human LHRH an LH.

Pan Vera has announced the availability of **anti-fluorescein single-chain antibody** (α -F1sFv) (*Reader Service No. 101*). Potential applications for this smaller sized (approximately 27-kD) molecule include fluorescence polarization and protein engineering studies, basic research of antibody-antigen binding characteristics, the study of tumour antigens, investigations in cell-specific immunotoxins and a broad range of clinical studies. It incorporates a linker peptide to bridge isolated immunoglobulin variable domains (V_L and V_H) into a single polypeptide. The recombinant protein is expressed in *Escherichia coli* within inclusion bodies, and the protein is denatured, refolded and purified to homogeneity. The manufacturer states that α -F1sFv retains very high binding affinities, comparable to those of the monoclonal antibodies from which it was derived. The sFv fragment is said to represent the minimal structural components that retain the equivalent binding-site characteristics of the parent antibody.



Pan Vera anti-fluorescence MAb.

Recombinant human and recombinant rat ciliary neurotrophic factor (CNTF) are now available from R&D Systems (*Reader Service No. 102*). These CNTF proteins have been measured in human erythroleukaemic cell line (TF-1) bioassays and tested for their ability to support the survival and neurite outgrowth of cultured embryonic chick



R&D neurotrophic factors and antibodies.

dorsal root ganglia. Two anti-CNTF antibodies are also available: polyclonal and monoclonal anti-human CNTF. There is also recombinant human soluble gp130 protein and both monoclonal and polyclonal anti-human gp130 antibodies. The anti-gp130 antibodies block cell-surface human gp130 mediated bioactivities induced by IL-6, IL-11, LIF and OSM in addition to CNTF.

■ Monoclonal antibodies

Transduction Laboratories has introduced a new line of **monoclonal antibodies to Stats 1–6** (IL-4 Stat) (*Reader Service No. 103*). These reagents are useful in western blotting, as well as in other immunological applications. Stats 1 and 2 both contain SH2 and SH3 domains and have been described as members of the ISGF3 (interferon-stimulated gene factor 3) complex. Stat 3 is activated as a DNA binding protein through tyrosine phosphorylation and can bind to DNA in the absence of Stat 1 α or Stat 2. Stat 4, like other Stat proteins, requires tyrosine phosphorylation in order to stimulate its DNA binding activity. However, its tissue distribution is much more limited. Stat5 (MFG) contains an SH2 domain and, once phosphorylated, acts as a transcription factor. Stat 6, also known as IL-4 Stat, appears to associate with the phosphorylated IL-4 receptor and plays a key role in IL-4 mediated signalling. Antibodies are available in 50 μ g and 150 μ g sizes. A Stat 1 antibody is also available as an agarose conjugate.

Berkeley Antibody has produced a **monoclonal antibody for the IL-2 receptor** (*Reader Service No. 104*). The massive proliferation of T cells during an antigenic response is initiated when interleukin-2 (IL-2), binds the IL-2 receptor, a 60-kD protein on the T-cell surface. This interaction is crucial for initiating the proliferation of T cells during an immune response, and is an important area of study for understanding the immune system's regulatory control pathways. The manufacturer offers monoclonal antibodies against the 55-kD α subunit of the human IL-2 receptor. This monoclonal antibody is said to be highly specific for its antigen and is suitable for various immunodetection assays, including immunoprecipitation and immunohistochemistry studies.

Kamiya Biomedical is now offering a complete line of anti-human and anti-mouse Fas/APO-1 [CD95] **monoclonal antibodies for apoptosis research** (*Reader Service No. 105*). Fas/APO-1 is a cell-surface receptor that can mediate apoptosis or programmed cell death. Three anti-human Fas monoclonals are available: clone CH-11 for apoptosis induction, immunoblotting and flow cytometry; clone UB2 for immunohistochemistry and flow cytometry; and clone ZB4 for blocking apoptosis, immunoblotting, immunohistochemistry and immunoprecipitation. Three anti-mouse Fas monoclonals are also available: clone RK8 is for inducing apoptosis and flow cytometry, clone RMF2 for inducing apoptosis flow and cytometry, and clone RMF6 for immunohistochemistry and flow cytometry. These six clones should prove useful for applications in cancer and cell biology research.

Eastman Kodak now offers IBI anti-FLAG BioM2 **monoclonal antibody for rapid detection of tagged recombinant proteins** (*Reader Service No. 106*). For detection of FLAG-tagged recombinant proteins, this monoclonal antibody should prove useful in western blot, ELISA and light-microscopic immunological assays. The product is a biotinylated version of the anti-FLAG M2 monoclonal antibody and can be detected by streptavidin- or avidin-conjugated enzymes such as streptavidin-HRP. It can bind a FLAG peptide that is fused to a cloned protein expressed by an animal, yeast, insect or *Escherichia coli* FLAG-expression vector. The product is available through a number of scientific supply companies.

■ Immunohistochemistry

Nanoprobes has announced what may prove to be a valuable new tool for immunohistochemistry, FAB-3 — a **3-nm colloidal gold particle conjugated to Fab' antibody fragments** (*Reader Service No. 107*). The small size of the resulting probe (smaller than a single IgG) is designed to allow penetration into tissues and better saturation of antigens. Smaller probes have been shown to give more quantitative antigen labelling. The 3-nm particle may be visualized directly at high magnification in the electron microscope, or silver enhanced for low-magnification

ACTH IRMA allows for clearer differentiation of normal ACTH and ACTH-suppressed levels. It is stated to be a highly specific and sensitive IRMA for the measurement of human ACTH 1–39. This is accomplished using two antibodies, each specific for a different region of the ACTH molecule. The iodinated tracer used in this system contains a purified polyclonal goat antibody specific for ACTH 26–39 and an ^{125}I -labelled monoclonal antibody specific for ACTH 1–17. The standards in the ACTH IRMA contain purified synthetic human ACTH. The assay is said to offer a sensitivity of 1.5 pg ml^{-1} and have little or no cross-reactivity with other peptides including ACTH 1–26, 18–24, 26–30, 1–10, α -MSH, β -MSH and β -endorphin.

■ New substrates

A new **peroxidase substrate** designed to offer significant advantages over DAB or enhanced DAB systems is now available from Kirkegaard & Perry Laboratories (*Reader Service No. 110*). TrueBlue peroxidase substrate produces a permanent blue stain that provides high-contrast and resolution of cellular detail, and is designed for multiple staining procedures.

Increased sensitivity is intended to offer substantial cost-per-test savings by allowing the use of higher primary antibody dilutions. TrueBlue is a stable, ready-to-use one-component formulation. It is not a carcinogen and requires no special disposal procedures. The product is part of the HistoMark line of immunohistostaining systems.

Immuno Concepts has developed a new ANA substrate that should increase the **detection of SS-A autoantibodies** (*Reader Service No. 111*). Hep-2000 are typical Hep-2 cells that have undergone a unique transfection process that results in the overexpression of the Ro/SS-A autoantigen. This allows preparation of Hep-2000 to detect much lower levels of anti-Ro/SS-A antibodies than cells that have not been transfected. Over-expressing cells show clear patterns and are easy to identify.

The new ELISA product range from Bionostics now includes Megga-Block 3, a new product for the **blocking of solid-phase enzyme linked immunosorbent assays** (*Reader Service No. 112*). The manufacturer states that the product has been formulated from defined components of small molecular size and should therefore prove to be an advance over traditional blocking materials such as bovine serum albumin or dried milk in terms of

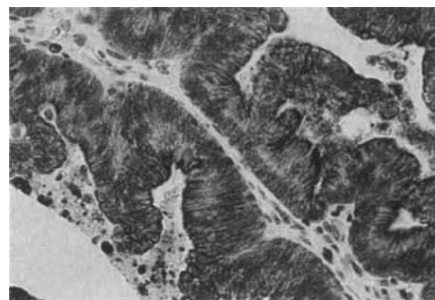
consistency, reproducibility and sensitivity. It does not exhibit the steric hindrance of the probe-target recognition associated with large molecular weight materials, and therefore enables a high ratio of signal to background. Megga-Block 3 is available in volumes suitable for research and manufacturing applications.

Designed to be an alternative to the alkaline phosphatase anti-alkaline phosphatase technique using rat monoclonal antibody, Life Science International now offers a new **detection technique for rat monoclonal antibodies** (*Reader Service No. 113*). Shandon Sequenza can be used for compounds that are difficult to stain: 2B-4, 1H-4 (anti-EBV EBNA-1) and SR109 (anti-HPV16E4), in both frozen and formalin fixed, paraffin-embedded tissues. Background staining is stated to be minimal due in part to the use of normal sheep serum as a diluent.

■ Cell study

Pharmacia has introduced new antibodies against human perforin and granzyme B for studies of cytolytic function and apoptosis (*Reader Service No. 114*). These proteins are contained in cytoplasmic granules of cytotoxic T lymphocytes and NK cells and have particular relevance to research on allograft rejection, graft-versus-host disease and T-cell mediated autoimmune diseases. Antibodies are available as ascites, purified, conjugated to biotin or coupled to TITC for ELISA, western-blot, immunohistochemical staining (frozen and paraffin-embedded tissue sections) and flow cytometry analysis.

Dako now offers an extensive list of **cytokeratin antibodies** for use in immunohistochemistry and flow cytometry (*Reader Service No. 115*). These include a variety of

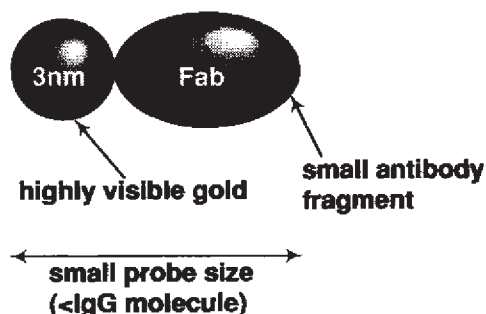


Dako Cytokeratin.

pan-screening, and high and low molecular weight cytokeratins such as AE1/AE3, 34 β E12, 35 β H11, cytokeratins 7, 8, 10/13, and 17–20, as well as others. Dako will furnish a complete list of the cytokeratin antibodies on request.

For **haematology, flow cytometry and blood cell analysis products**, Coulter offers PCNA-FITC, CD15-RD1 and gly-

FAB-3



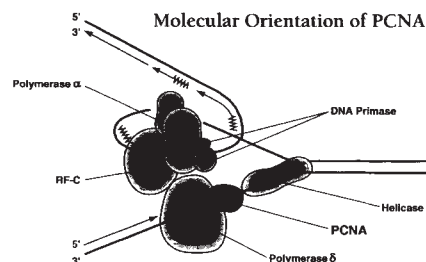
Nanoprobes small-size gold particle-Fab probe

work and light and confocal microscopy applications. FAB-3 is gel filtered to eliminate aggregates and is now available with goat anti-mouse, rabbit anti-goat, and other commonly used secondary antibodies, as well as anti-biotin Fab'.

BioGenex has developed a **monoclonal antibody to bcl-2 oncoprotein** for immunohistochemical analysis of human tissue sections (*Reader Service No. 108*). The *bcl-2* oncoprotein, an integral protein of the mitochondrial inner membrane, is frequently overexpressed in lymphoid malignancies. This monoclonal antibody can be used after microwave pretreatment with Antigen Retrieval Citra solution, it produces intense staining of *bcl-2* in paraffin-embedded tissues. The anti-*bcl-2* monoclonal antibody reacts strongly with follicular lymphoma, hairy cell leukemia, high-grade B-cell and T-cell lymphomas, lymphoblastic lymphoma and 'Ki-1 lymphoma' (anaplastic large-cell lymphoma). The antibody also reacts with small lymphocytes in T-cell areas and mantle zones in the tonsils and lymph nodes. It does not react with cells in the germinal centres of lymphoid tissues.

Incstar now offers an immunoradiometric assay (IRMA) for the **measurement of human ACTH** (*Reader Service No. 109*). The company states that the

cophorin A-RD1 (*Reader Service No. 116*). All three research-grade products are packaged in liquid-conjugated form and can be used directly from the vial for flow cytometric procedures. PCNA is useful in certain tissue and blood studies. CD15-RD1 can be used in white blood cell studies and glycophorin and A-RD1 should prove useful in red blood cell studies. PCNA-FITC is a monoclonal antibody directed against proliferating cell



PCNA-FITC from Coulter.

nuclear antigen. PCNA is a 36-kD intranuclear polypeptide closely associated with the sites of DNA replication. It is the auxiliary protein to DNA polymerase-delta and is found in normal and transformed cells. Elevated levels of PCNA appear in the late G1 phase of the cell cycle, maximizing during S phase and declining during G2 and M phases. The level of PCNA directly correlates with cellular proliferation, specifically DNA synthesis during the cell cycle. CD15-RD1 is a monoclonal antibody that detects the CD15 antigen expressed on granulocyte-committed cells. CD15 RD1 is moderately expressed on monocytes and strongly expressed on neutrophils and eosinophils, but does not react with other white blood cell populations. A-RD1 is a monoclonal antibody specific to red blood cells and erythroid precursor cells from erythroblast to cell maturity. It does not cross-react with glycophorin B or C, or with white blood cells.

Sigma Immunochemicals has released four new **monoclonal antibodies to adaptins** and **two new monoclonal antibodies to Golgi proteins** (*Reader Service No. 117*). These are intended for use in cell biology studies for protein identification and to assist in understanding the roles and relationships of the proteins in the golgi apparatus. The monoclonal anti-adaptins offer the ability to identify intracellular adaptor proteins of the Golgi-adaptor complex. With the exception of the anti- α -adaptin, these monoclonal antibodies may be used in immunocytochemistry of intact cells. The titre ranges are from 1:1,000 to 1:10,000 in immunoblotting. The monoclonal anti-Golgi proteins available are anti-Golgi β -COP protein (clone M3A5) and anti-Golgi 58K protein (clone 58K-9). These antibodies cross-react with

a variety of species including human and rat, and may be used in immunocytochemistry and immunoblotting as well as other applications. Titres range from 1:20 to 1:50 in immunofluorescence and 1:50 to 1:5,000 in immunoblotting.

TCS Biologicals now offers several monoclonal antibodies for **studying the expression, distribution and function of gp170** (p-170 glycoprotein) (*Reader Service No. 118*). This product of the MDR-1 gene is often overexpressed in cancer patients and is associated with multi-drug resistance to anticancer drugs. This protein functions as a pleiotropic transport protein, which uses ATP hydrolysis to drive the expulsion of toxic molecules, including many chemotherapeutic agents, from the cell. The company offers several monoclonal antibodies for this area: clone MRK 16 is specific for a cell surface epitope of human gp170 and is recommended for FACS analysis; clone JSB-1 reacts with a strongly conserved cytoplasmic epitope; and clone LRP-56 is specific to human cell-surface glycoprotein, called p100, which is also associated with MDR.

Microplate equipment

Labsystems has designed the new Thermomix **incubator/shaker** to cover a temperature range from 3 °C to 70 °C (*Reader Service No. 119*). This apparatus is stated to have a temperature uniformity of ≤ 0.6 °C and temperature accuracy of ± 0.5 °C. It has a capacity of three microplates and has incorporated an orbital shaker with five speeds. Thermomix should prove useful in different molecular biological and biochemical assays.



Thermomix from Labsystems.

Denley has launched a new **microplate washer with orbital shaking** called Aquarius (*Reader Service No. 120*). The device is automatically optimized according to dispense volume. The duration can be programmed as part of the assay protocol. All wash parameters, including dispense volume, plate or strip, soak time, dispense height and aspirate height are user programmable through a simple four-key menu tree contained within the programme module. A total of 99 individual programmes can be named and stored. It also has a multi-buffer system that allows two 2-litre reagent bottles, one 1-litre rinse bottle and a one 4.5-litre waste bottle to be switched through an electronically operated valve. The rinse cycle can be automatically set to rinse at regular intervals and a spare air channel is available to completely purge the system. All reagent and waste bottles have liquid sensors, which operate audio and visual warnings.

For a precision **microplate instrument** specifically designed to simplify immunoassay procedures, Bio-Tek Instruments offers the Ceres 900C (*Reader Service No. 121*). This instrument is intended for use in end point and kinetic ELISAs with scanning capabilities for determining agglutination patterns and cell

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BioTek Ceres 900C.

counting. The 900C operates in manual and automated modes, has a modular design and runs under an IBM PC or compatible, or Macintosh software, environment. Applications include ELISA, protein determinations, cytotoxicity, cell growth, MIC determinations and agglutination assays.

■ ELISA

In response to the growing emphasis on *in vitro* metabolism studies for new drug testing, Amersham Life Science has developed four **cytochrome P-450 isoenzyme ELISAs**, and six ¹⁴C-labelled cytochrome P450 substrates (*Reader Service No. 122*). The new ELISAs will be useful for determining not only whether a drug has induced a particular cytochrome P-450 isoform, but also how much induction has occurred. Each ELISA uses purified antibodies enabling specific detection of the 1A1, 2B1/2, 3A and 4A isoforms of cytochrome P-450 in rat liver microsomal samples. The radiolabel has been incorporated into a metabolically stable position that enables any metabolites to be identified. The six new substrates are [1-¹⁴C]lauric acid, [4-¹⁴C]testosterone, [2-¹⁴C]chlorozoxazone, [S-¹⁴C]mephentoin, [guanidine-¹⁴C]debrisoquine and [carbonyl-¹⁴C]tolbutamide.

The soluble **ICAM-1 and CD23 ELISA kits**, by Serotec, have been designed for high-sensitivity recognition of very low amounts of these antigen (*Reader Service No. 123*). The soluble ICAM-1 kit should prove to be of importance in the early detection of rejection of liver transplants, sepsis in the neonate, rheumatoid arthritis and various malignant diseases of the adult. The manufacturer states that the normal range of serum sICAM-1 is 100–200 ng ml⁻¹. Levels can rise to 1,500 ng ml⁻¹ in patients acutely rejecting liver transplants. The kit contains a 96-well precoated microtitre plate, standards, second antibody and substrate. CD23, also known as the IgE Fc low-affinity receptor, is a cell-surface protein expressed by activated B cells, activated macrophages, eosinophils and platelets. Soluble CD23, also known as B-cell growth factor (BCGF), has been implicated in B-cell-derived chronic lympho-

cytic leukaemia, autoimmune chronic active hepatitis and hairy cell eosinophilic syndrome. The CD23 ELISA kit is sensitive to 1.3 units ml⁻¹ in a total incubation period of 2 h 50 min, and is supplied with a 96-well precoated microtitre plate, standards, anti-sCD23 conjugated to horseradish peroxidase and all reagents necessary to develop the staining.

■ Miscellaneous

Biocytex-Bioservices specializes in developing and producing **polyclonal antibodies** in various animal species such as rabbit, chicken, goat and rat (*Reader Service No. 124*). Animals are boarded in-house in a controlled environment. A programme can involve antigens such as proteins, chemicals, drugs, bacterial and viral antigens. Anti-peptide antibody production is also available with custom peptide synthesis. These polyclonal antibodies are supplied as antisera or purified. Secondary antibodies are also offered. Scale-up contract production is available as well as a comprehensive immunoassay development programme.

New **antibodies** are now available from NBS Biologicals to give researchers in geriatrics, neuroscience and neuropharmacology more tools to extend their knowledge of the precursors surrounding Alzheimer's disease (*Reader Service No. 125*). The new antibodies range from monoclonal mouse anti-tau AT8 through to recombinant human tau to MAP2 and GAP43. Data sheets for these products can be obtained from NBS Biologicals.

Autogen Bioclear offers a new **retroviral and cytokine products catalogue** from Intracel listing a wide range of products for retroviral research, including recombinant proteins, monoclonal and polyclonal antibodies, peptides and capture ELISA kits (*Reader Service No. 126*). This new range may prove to be an invaluable tool for research in HIV-1, HIV-2, HTLV-1, HTLV-2, SIV or T-cell receptors.

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

CORRECTION

For the issue 6 April 1995 on page 573 read in part, "scop.mrcimb.cam.ac.uk/scop" and should have read in part, "scop.mrc-lmb.cam.ac.uk/scop". Also, "http://ncbi.nlm.nih.gov/repository/scop/index.html" should have read, "ftp://ncbi.nlm.nih.gov/repository/scop/index.html".

ERRATUM

The last sentence of Oxford Glycosystems' recombinant phosphatidyl inositol-specific phospholipase C (PI-PLC) product review (*Nature* 374 389; 23 March 1995), should have read, "A number of different pack sizes is available" in the last line.

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